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Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Method for producing nucleoside-5'-phosphate ester

(57) A method for producing nucleoside-5'-phosphate ester involves phosphorylating nucleoside biochemically.

Nucleoside-5'-phosphate ester is produced by allowing an acid phosphatase, especially an acid phosphatase having an increased affinity for a nucleoside and/or an increased temperature stability, to act at pH 3.0 to 5.5 on a nucleoside and a phosphate group donor.

The phosphate group donor may be selected from polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, and carbamyl phosphate or a salt thereof. The resulting nucleoside-5'-phosphate ester may subsequently be collected. As an alternative to using isolated acid phosphatase to catalyse the reaction, a microorganism transformed with a gene encoding a protein having acid phosphatase activity may be employed.

Description

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The present invention relates to a method for producing nucleoside-5'-phosphate ester. The present invention also relates to a novel acid phosphatase, a gene coding for the acid phosphatase, a recombinant DNA containing the gene, and a microorganism harboring the recombinant DNA which are useful to produce nucleoside-5'-phosphate ester. Nucleoside-5'-phosphate ester is useful as a seasoning, a pharmaceutical, and a raw material for producing such substances.

Methods for biochemically phosphorylating nucleoside to produce nucleoside-5'-phosphate ester by using the following phosphate group donors are known, including a method which uses p-nitrophenyphosphoric acid (Japanese Patent Publication No. 39-29858), a method which uses inorganic phosphoric acid (Japanese Patent Publication No. 42-1186), a method which uses polyphosphoric acid (Japanese Patent Laid-open No. 53-56390), a method which uses acetylphosphoric acid (Japanese Patent Laid-open No. 56-82098), and a method which uses adenosine triphosphate (ATP) (Japanese Patent Laid-open No. 63-230094). However, these methods have not been satisfactory to produce nucleoside-5'-phosphate ester efficiently and inexpensively because the substrates to be used are expensive, or because by-products are produced in the reaction.

Thus the present inventors have developed a method for efficiently producing nucleoside-5'-phosphate ester without by-producing 2'-, 3'-nucleotide isomers by allowing cells of a specified microorganism to act under an acidic condition on a nucleoside and a phosphate group donor selected from the group consisting of polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, and carbamyl phosphate or a salt thereof (Japanese Patent Laidopen No. 7-231793).

However, even this method has had the following drawbacks. Namely, for example, a part of the substrate is degraded during the reaction due to a nucleoside-degrading activity which unfortunately exists in a slight amount in the cells of the microorganism to be used. Moreover, if the reaction is continued, produced and accumulated nucleoside-5'-phosphate ester is degraded. Therefore, by-products are produced in a reaction solution, and it has been impossible to obtain a sufficient yield. In addition, the reaction cannot be performed if the substrate is added at a high concentration because of a low transphosphorylation activity per microbial cell.

In one embodiment, the present invention desirably provides a method for inexpensively and efficiently producing nucleoside-5'-phosphate ester. According to other embodiments of the present invention there are provided an enzyme, a gene coding for the enzyme, a recombinant DNA containing the gene, and a microorganism harboring the recombinant DNA which are useful for the method for producing nucleoside-5'-phosphate ester.

As a result of various investigations made by the present inventors in order to develop a method for producing nucleoside-5'-phosphate ester which is more efficient than the conventional methods, it has been found out that nucleoside-5'-phosphate ester maybe efficiently produced at a high yield by allowing an acid phosphatase purified from a cell-free extract of a microorganism to act under a condition of pH 3.0 to 5.5 on a nucleoside and a phosphate group donor selected from the group consisting of polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, and carbamyl phosphate or a salt thereof. Further, the present inventors have succeeded in obtaining wild type genes coding for acid phosphatases from various bacteria and genes coding for acid phosphatases having an increased affinity for the nucleoside as compared with the wild type acid phophatase by introducing a mutation into an acid phosphatase derived from a bacterium belonging to the genus Escherichia. Moreover, the present inventors have found out that productivity of nucleoside-5'-phosphate ester may be remarkably improved by expressing the gene in a large amount in accordance with genetic engineering techniques.

Further the present inventors have tried to prepare a mutant acid phosphatase with an increased temperature stability, with the intention that conducting a phosphate transfer reaction using the acid phosphatase at a higher temperature leads to a much more effective production of nucleoside-5'-phosphate because the reaction speed is elevated and the concentration of phosphate receiver in the reaction solution can be higher. Then the present inventors have succeeded in the preparation of a mutant acid phosphatase which has an increased temperature stability over mutant acid phosphatases described in Example 19 and can be employed in a reaction at a high temperature, and completed the present invention

Namely, the present invention provides a method for producing nucleoside-5'-phosphate ester comprising the steps of allowing an acid phosphatase having an increased affinity for a nucleoside and/or an increased temperature stability to act under a condition of pH 3.0 to 5.5 on a nucleoside and a phosphate group donor, preferably selected from the group consisting of polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, acetylphosphoric acid or a salt thereof, and carbamyl phosphate or a salt thereof to produce nucleoside-5'-phosphate ester, and collecting it.

The term "acid phosphatase having an increased affinity for nucleoside" encompasses those acid phosphatases in which the affinity for nucleoside is higher in comparison with the wild type acid phosphatase. Preferred acid phosphatases have a Michaelis constant, Km, for transphosphorylation of the nucleoside, which is below 100mM.

The term "acid phosphatase having increase temperature stability" encompasses those acid phosphatases which

retain more residual activity after treatment at elevated temperature than does a corresponding wild type acid phosphatase. Preferably, the acid phosphatase retains more activity than the wild type after being held at 50°C for 30 minutes at pH 7.0. Particularly preferably, the acid phosphatase shows substantially no decrease in activity on treatment for 30 minutes at 50°C and pH 7.0.

In another aspect, the present invention provides a method for producing nucleoside-5'-phosphate ester comprising the steps of allowing a microorganism to act under a condition of pH 3.0 to 5.5 on a nucleoside and a phosphate group donor to produce nucleoside -5'-phosphate ester, and collecting it, wherein the microorgansm is transformed with a recombinant DNA comprising a gene encoding an acid phosphate having an increased affinity for the nucleoside and/ or an increased temperature affinity.

In another aspect, the present invention provides mutant acid phosphatases having an increased affinity for a nucleoside and/or an increased temperature stability, genes coding for the acid phosphatases, recombinant DNAs containing the genes, and microorganisms harboring the recombinant DNA.

Embodiments of the invention are described below by way of example only and with reference to the accompanying figures, of which:

- Fig. 1 illustrates a relationship between reaction pH and produced amount of 5'-inosinic acid in a reaction performed by using an enzyme derived from Morganella morganii.
- Fig. 2 illustrates a relationship between reaction pH and produced amount of 5'-inosinic acid in a reaction performed by using an enzyme derived from Escherichia blattae.
- Fig. 3 illustrates a restriction enzyme map of a chromosomal DNA fragment of Morganella morganii containing a gene coding for an acid phosphatase.
- Fig. 4 illustrates produced amount of 5'-inosinic acid in a reaction performed by using a strain harboring phosphatase gene derived from Morganella morganii.
- Fig. 5 illustrates a restriction enzyme map of a chromosomal DNA fragment of Escherichia blattae containing a gene coding for an acid phosphatase.
- Fig. 6 illustrates a diagram showing produced amount of 5'-inosinic acid in a reaction performed by using a strain harboring the acid phosphatase gene derived from Escherichia blattae.
- Fig. 7 illustrates produced amount of 5'-inosinic acid in reactions performed by using a strain harboring the wild type acid phosphatase gene and a strain harboring the mutant acid phosphatase gene derived from Escherichia blattae respectively.
- Fig. 8 illustrates produced amount of 5'-inosinic acid in a reaction performed by using a strain harboring the new mutant phosphatase gene derived from <u>Escherichia blattae</u>.
- Fig. 9 illustrates a restriction enzyme map of a chromosomal DNA fragment derived from Enterobacter aerogenes which contains the gene coding for acid phosphatase.
- Fig. 10 illustrates a restriction enzyme map of a chromosomal DNA fragment derived from Klebsiella planticola which contains the gene coding for acid phosphatase.
- Fig. 11 illustrates a restriction enzyme map of a chromosomal DNA fragment derived from <u>Serratia</u> <u>ficaria</u> which contains the gene coding for acid phosphatase.
- Fig. 12 illustrates amino acid sequences in one-letter deduced from nucleotide sequences of acid phosphatases derived from Morganella morganii, Escherichia blattae, Providencia stuartii, Enterobacter aerogenes, Klebsiella planticola and Serratia ficaria. These amino acid sequences are illustrated in SEQ ID NOs: 4, 8, 22, 24, 26 and 28 in Sequence Listing in three-letter. In the figuer, the amino acid residues which is common through the all amino acid sequences are marked with * below the sequence.
- Fig. 13 illustrates the graph of the temperature stability of the acid phosphatase activity in the cell free extract solution prepared from a strain harboring the new mutant phosphatase gene derived from Escherichia blattae.

<1> Preparation of acid phosphatase

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The acid phosphatase to be used in the present invention is not specifically limited provided that it catalyzes the reaction to produce nucleoside-5'-phosphate ester by phosphate group transfer to the nucleoside from the phosphate group donor, for example, selected from the group consisting of polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, acethylphosphoric acid or a salt, and carbamyl phosphate or a salt thereof under the condition of pH 3.0 to 5.5. Such an acid phosphatase preferably includes those derived from microorganisms. In an especially preferred embodiment, the present invention uses an enzyme derived from a bacterium belonging to the genus Morganella, Escherichia, Providencia,

Enterobacter, Klebsiella or Serratia. Representative examples of such a bacterium include the following bacterial strains.

Moganella morganii NCIMB 10466

Morganella morganii IFO 3168
Morganella morganii IFO 3848
Escherichia blattae JCM 1650
Escherichia blattae ATCC 33429
Escherichia blattae ATCC 33430
Providencia stuartii ATCC 29851
Providencia stuartii ATCC 33672
Enterobacter aerogenes IFO 12010
Enterobacter aerogenes IFO 13534
Klebsiella planticola IFO 14939
Klebsiella planticola IAM 1133
Serratia ficaria IAM 13540
Serratia marcescens IAM 12143

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It is noted that acid phosphatase (EC 3.1.3.2) is originally an enzyme which catalyzes a reaction to hydrolyze phosphate ester under an acidic condition, and it has a nucleotidase activity to degrade nucleoside-5'-phosphate ester produced by the transphosphorylation reaction (hereinafter, the nucleotidase activity is referred to as "phosphomonoesterase activity"). Even such an acid phosphatase can be used in the method for producing nucleoside-5'-phosphate ester of the present invention. However, in order to obtain nucleoside-5'-phosphate ester at a high yield, it is desirable to use the mutant acid phosphatase in which an affinity for a nucleoside in the transphosphorylation reaction onto the nucleoside is increased as compared with the wild type acid phosphatase produced by the bacteria as described above (hereinafter simply referred to as "mutant acid phosphatase", if necessary). Preferably the mutant acid phosphatase having a Km value below 100 is used.

The mutant acid phosphatase may be obtained by expressing a mutant gene obtained by directly mutating a gene coding for an acid phosphatase as described below. Alternatively, the mutant acid phosphatase may be also obtained by treating a microorganism which produces an acid phosphatase with irradiation of ultraviolet light or a mutating agent usually used for artificial mutation such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and cultivating a microorganism mutated to produce a mutant acid phosphatase having an increased affinity for a nucleoside and/or an increased temperature stability.

A protein having the acid phosphatase activity may be obtained from the microorganisms as described above by cultivating the microbial strain having the activity in an appropriate medium, harvesting proliferated microbial cells, disrupting the microbial cells to prepare a cell-free extract, and adequately purifying the protein therefrom.

The medium for cultivating the microorganism is not specifically limited, for which an ordinary medium may be available, containing an ordinary carbon source, a nitrogen source, inorganic ions, and optionally an organic nutrient source. The carbon source to be adequately used includes, for example, saccharides such as glucose and sucrose, alcohols such as glycerol, and organic acids. The nitrogen source to be used includes, for example, ammonia gas, aqueous ammonia, and ammonium salts. The inorganic ions to be adequately used if necessary include, for example, magnesium ion, phosphate ion, potassium ion, iron ion, and manganese ion. The organic nutrient source to be adequately used includes, for example, vitamins and amino acids as well as those containing them such as yeast extract, peptone, meat extract, corn steep liquor, casein hydrolysate, and soybean hydrolysate.

The cultivation condition is also not specifically limited. The microorganism may be cultivated, for example, under an aerobic condition for about 12 to 48 hours while appropriately controlling pH and temperature within ranges of pH 5 to 8 and temperature of 25 to 40 °C.

Proliferated microbial cells may be harvested from a culture liquid, for example, by centrifugation. The cell-free extract is prepared from the harvested microbial cells by using an ordinary method. Namely, the cell-free extract is obtained by disrupting the microbial cells by means of a method such as ultrasonic treatment, Dyno-mill, and French Press, and removing cell debris by centrifugation.

The acid phosphatase is purified from the cell-free extract by using an adequate combination of techniques usually used for enzyme purification such as ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, gel filtration chromatography, and isoelectric purification. As for the precipitation, it is not necessarily indispensable to completely purify the acid phosphatase. It is sufficient to achieve removal of contaminants such as an enzyme which participates in degradation of nucleoside as the substrate.

<2> Preparation of acid phosphatase gene

A DNA fragment, which contains a structural gene coding for the protein having the acid phosphatase activity, can be cloned starting from, for example, cells of the microorganism having the enzyme activity. The cloning method includes, for example, a method in which a chromosomal gene expression library is screened by using the enzyme

activity as an index, a method in which an antibody against the protein is prepared to screen a chromosomal gene expression library, and a method in which an amino acid sequence such as an N-terminal sequence of the purified protein is analyzed, on the basis of which a probe is prepared to screen a gene library.

Specifically, the gene coding for the acid phosphatase of Morganella morganii, Escherichia blattae, Providencia stuartii, Enterobacter aerogenes, Klebsiella planticola, Serratia ficaria or Serratia marcescens described above can be cloned by preparing a chromosomal gene expression library of each of the microorganisms, and screening the library by using the phosphatase activity as an index.

Namely, a chromosomal gene expression library can be prepared by firstly preparing chromosomal DNA from Morganella morganii or Escherichia blattae, partially degrading it with an appropriate restriction enzyme, subsequently ligating it with a vector autonomously replicable in Escherichia coli, and transforming Escherichia coli with the obtained recombinant DNA. A wide variety of restriction enzymes can be used for digesting chromosomal DNA by adjusting the digestion reaction time to adjust the degree of digestion. Any vector may be used for cloning the gene provided that it is autonomously replicable in Escherichia coli. It is possible to use, for example, pUC19, pUC118, pHSG298, pBR322, and pBluescript II.

The vector may be ligated with the DNA fragment containing the gene coding for the acid phosphatase to prepare the recombinant DNA by previously digesting the vector with the same restriction enzyme as that used for digesting chromosomal DNA, or with a restriction enzyme which generates a cleaved edge complementary with a cleaved edge of the chromosomal DNA fragment, and ligating it with the DNA fragment by using ligase such as T4 DNA ligase. Any microbial strain may be used as a recipient for the prepared recombinant DNA provided that it is appropriate for replication of the vector. It is possible to use, for example, microbial strains of Escherichia coli such as HB101, JM109, and DH5.

Transformants thus obtained are grown on an agar medium to form their colonies. After that, when a reaction solution containing p-nitrophenylphosphoric acid is poured onto a surface of the medium to perform a reaction, then a strain, which has expressed the phosphatase activity, liberates p-nitrophenol and exhibits a yellow color. A transformant, which harbors a DNA fragment containing the gene coding for the objective acid phosphatase, can be selected by performing the reaction described above under an acidic condition, and selecting the transformant by using the color development as an index.

After that, a recombinant DNA is recovered from the selected transformant to analyze the structure of the DNA fragment containing the gene coding for the acid phosphatase ligated with the vector. A nucleotide sequence of the gene coding for the acid phosphatase is shown in SEQ ID NO: 2 in Sequence Listing in the case of a gene derived from Morganella morganii NCIMB 10466, SEQ ID NO: 6 in Sequence Listing in the case of a gene derived from Escherichia blattae JCM 1650, SEQ ID NO: 21 in Sequence Listing in the case of a gene derived from Providencia stuartii ATCC 29851, SEQ ID NO: 23 in Sequence Listing in the case of a gene derived from Enterobacter aerogenes IFO 12010, SEQ ID NO: 25 in Sequence Listing in the case of a gene derived from Klebsiella planticola IFO14939, or SEQ ID NO: 27 in Sequence Listing in the case of a gene derived from Serratia ficaria IAM 13540.

The deduced amino acid sequences of the acid phosphatases encoded by the above genes are illustrated in SEQ ID NO: 4, 8, 22, 24, 26 and 28.

Variants of the acid phosphatases encoded by the above genes and having increased affinity for nucleoside and/ or increased temperature stability, are preferably used for the present invention. Acid phosphatase comprising an amino acid sequence which is substantially identical with an amino acid sequence of any one of the acid phosphatases encoded by the above genes is also preferably used for the present invention. The term "substantially identical" means that amino acid sequences of the acid, phosphatases may have substitution, deletion, insertion or transition of one or a plurality of amino acid residues without losing an activity to produce nucleoside-5'-phosphate ester (hereinafter referred to as "transphosphorylation activity"). Preferably, the variants have a Michaelis constant Km below 100°C as discussed above and experience substantially no loss of activity when held at 50°C and pH7 for 30 minutes.

<3> Preparation of gene coding for mutant acid phosphatase

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The wild type acid phosphatase obtained as described above has a phosphomonoesterase activity. Therefore, the phosphomonoesterase activity may serve as a factor to cause accompanying degradation of the product as the reaction time passes in the production of nucleoside-5'-phosphate ester, resulting in decrease in reaction yield. In order to overcome such a circumstance, it is advantageous to cause artificial mutation on the gene coding for the acid phosphatase so that an affinity for a nucleoside is increased.

Further conducting a phosphate transfer reaction by the acid phosphatase at a higher temperature leads to a much more effective production of nucleoside-5'-phosphate because the reaction speed is elevated and the concentrattion of a phosphate reciever in the reaction solution can be higher. For the purpose it is advantageous to cause artificial mutation on the gene coding for the acid phosphatase so that a temperatre stability is increased.

Methods for site-directed mutagenesis for causing objective mutation at an objective site of DNA include, for ex-

ample, a method using PCR (Higuchi, R., 61, in <u>PCR technology</u>, Erlich, H. A. Eds., Stockton press (1989); carter, P., <u>Meth. in Enzymol.</u>, <u>154</u>, 382 (1987)), and a method using phage (Kramer, W. and Frits, H. J., <u>Meth. in Enzymol.</u>, <u>154</u>, 350 (1987); Kunkel, T. A. et al., <u>Meth. in Enzymol.</u>, <u>154</u>, 367 (1987)).

The mutant acid phosphatase having the increased affinity for the nucleoside is exemplified by the acid phosphatase comprising an amino acid sequence which is substantially identical with an amino acid sequence selected from the group consisting of sequences illustrated in SEQ ID NOs: 4, 8, 22, 24, 26 and 28 in Sequence Listing, and has mutation which increases the affinity for the nucleoside compared to the wild type acid phosphatase. Concretely, the mutant acid phosphatase is exemplified, for the enzyme derived from Escherichia blattae JCM 1650, by one in which the 74th glycine residue and/or the 153th isoleucine residue is substituted with another amino acid residue in an amino acid sequence illustrated in SEQ ID NO: 8 in Sequence Listing. In Examples described below, a gene coding for mutant acid phosphatase is illustrated as an example in which the 74th glycine residue is substituted with an aspartic acid residue, and the 153th isoleucine residue is substituted with a threonine residue.

Further mutations selected from the group consisting of substitutions of the 63rd leucine residue, the 65th alanine residue, the 66th glutamic acid residue, the 69th aspartic acid residue, the 71st serine residue, the 72nd serine residue, the 85th serine residue, the 92nd alanine residue, the 94th alanine residue, the 116th aspartic acid residue, the 130th serine residue, the 135th threonine residue and/or the 136th glutamic acid residue with another amino acid in SEQ ID NO: 8 in Sequence Listing further increase the affinity for the nucleoside of the acid phosphatase.

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The mutant acid phosphatase having the increased temperature stability is exemplified by the acid phosphatase comprising an amino acid sequence which is substantially identical with an amino acid sequence selected from the group consisting of sequences illustrated in SEQID NOs: 4, 8, 22, 24, 26 and 28 in Sequence Listing, and has mutation which increases the temperature stability of wild type acid phosphatase. Concretely, the mutant acid phosphatase is exemplified, for the enzyme derived from Escherichia blattae JCM 1650, by one in which the 104th glutamic acid residue and/or the 151st threonine residue is substituted with another amino acid residue in an amino acid sequence illustrated in SEQ ID NO: 8 in Sequence Listing. In Examples described below, a gene coding for mutant acid phosphatase is illustrated as an example in which the 104th glutamic acid residue is substituted with a glycine residue, and the 151st threonine residue is substituted with an alanine residue.

Therefore, the nucleotide may be substituted at the specified site of the wild type gene in accordance with the site-directed mutagenesis method described above so that these mutant acid phosphatases are encoded. The mutation to increase the affinity for the nucleoside is desirably a type of mutation by which the activity to produce nucleoside-5'-phosphate ester is not substantially lowered in comparison with wild type acid phosphatase. However, even in the case that the activity to produce nucleoside-5'-phosphate ester is lowered, it will be sufficient if degree of decrease of phosphomonoesterase activity is larger than that of the activity to produce nucleoside-5'-phosphate ester, with the result that a ratio of phosphomonoesterase activity to the activity to produce nucleoside-5'-phosphate ester of the mutant acid phosphatase is lowered in comparison with the wild type acid phosphatase. As for the degree of increase in the affinity for the nucleoside, the Km value to the nucleoside in the transphosphorylation reaction is preferably below 100.

The mutation which increases the temperature stability means one which has more residual activity after a temperature treatment than the wild type acid phosphatase has. The degree of the temperature stability increase is preferably the one that does not cause the decrease in an activity with the treatment at pH 7.0, 50°C, 30 minutes.

As illustrated below in the embodiments, the amino acid sequence of the acid phosphatase of Escherichia blattae JCM 1650 is highly homologous to that of Morganella morganii NCIMB 10466, and the 72nd glycine residue, the 102th glutamic acid residue, the 149th threonine residue and the 151th isoleucine residue in an amino acid sequence illustrated in SEQ ID NO: 4 correspond to the 74th glycine residue, the 104th glutamic acid residue, the 151st threonine residue and the 153rd isoleucine residue in an amino acid sequence illustrated in SEQID NO: 8 respectively. Further, in addition to Escherichia blattae JCM 1650, amino acid sequences of acid phosphatases derived from microorganisms such as Providencia stuartii ATCC 29851, Enterobacter aerogenes IFO 12010, Klebsiella planticola IFO 14939, and Serratia ficaria IAM 13450 have high homology with that of Morganella morganii NCIMB 10466, and amino acid sequences of these acid phosphatases include amino acids resudues each of which corresponds to the 72nd glycine residue, the 102nd glutamic acid residue, the 149th threonine residue and the 151st isoleucine residue in an amino acid sequence illustrated in SEQ ID NO: 4 respectively. Therefore, genes coding for mutant acid phosphatases derived from these microorganisms may be obtained as described above. The 92nd glycine residue, the 122nd glutamic acid residue, the 169th threonine residue and the 171st isoleucine residue in the amino acid sequence of the acid phosphatase derived from Providencia stuartii ATCC 29851, Enterobacter aerogenes IFO 12010 or Klebsiella planticola IFO 14939 illustrated in SEQ ID NO: 22, 24 or 26, and the 88th glycine residue, the 118th glutamic acid residue, the 165th threonine residue and the 167th isoleucine residue in the amino acid sequence of the acid phosphatase derived from Serratia ficaria IAM 13450 illustrated in SEQ ID NO: 28 respectively correspond to the 72nd glycine residue, the 102nd glutamic acid residue, the 149th threonine residue and the 151st isoleucine residue in an amino acid sequence illustrated in SEQ ID NO: 4.

The result of comparing the amino acid sequences of the above acid phophatase is illustrated in Fig. 12. Based on Fig. 12, it is decided which amino acid residue of one acid phosphatase corresponds to another amino acid residue of another acid phosphatase.

<4> Introduction of acid phosphatase gene into host

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A recombinant microorganism for expressing the acid phosphatase activity at a high level can be obtained by introducing the DNA fragment containing the gene coding for the protein having the acid phosphatase activity obtained as described above into cells of a host after recombining the DNA fragment again with an appropriate vector. In such a procedure, the wild type acid phosphatase is expressed by using the gene coding for the wild type acid phosphatase, while the mutant acid phosphatase is expressed by using the gene coding for the mutant acid phosphatase.

The host includes the microbial strains of <u>Escherichia coli</u> such as HB101, JM109, and DH5 described above. Other than these strains, all bacteria can be utilized as the host provided that a replication origin of constructed recombinant DNA and the acid phosphatase gene make their functions, the recombinant DNA is replicable, and the acid phosphatase gene is expressible. One of the most preferred hosts is <u>Escherichia coli</u> JM109.

The vector for incorporating the gene coding for the acid phosphatase thereinto is not specifically limited provided that it is replicable in the host. When Escherichia coli is used as the host, the vector may be exemplified by plasmids autonomously replicable in this bacterium. For example, it is possible to use ColE1 type plasmids, p15A type plasmids, R factor type plasmids, and phage type plasmids. Such plasmids specifically include, for example, pBR322 (Gene, 2, 95 (1977)), pUC19 (Gene, 33, 103 (1985)), pUC119 (Methods in Enzymology, 153, 3 (1987)), pACYC184 (J. Bacteriol., 134, 1141 (1978)), and pSC101 (Proc. Natl. Acad. Sci. U.S.A., 70, 3240 (1973)).

When the DNA fragment containing the gene coding for the acid phosphatase contains a promoter which is functional in the host, the DNA fragment may be ligated with the vector as it is. When the DNA fragment does not contain such a promoter, another promoter which works in the host microorganism such as lac, trp, and PL may be ligated at a position upstream from the gene. Even when the DNA fragment contains the promoter, the promoter may be substituted with another promoter in order to efficiently express the gene coding for the acid phosphatase.

There is no special limitation for a method for introducing, into the host, the recombinant DNA constructed by ligating the vector with the DNA fragment containing the gene coding for the acid phosphatase. The recombinant DNA may be introduced into the host by using an ordinary method. When Escherichia coli is used as the host, it is possible to use, for example, a calcium chloride method (J. Mol. Biol., 53, 159 (1970)), a method of Hanahan (J. Mol. Biol., 166, 557 (1983)), an SEM method (Gene, 96, 23 (1990)), a method of Chung et al. (Proc. Natl. Acad. Sci. U.S.A., 86, 2172 (1989)), and electroporation (Nucleic Acids Res., 16, 6127 (1988)).

The acid phosphatase gene may be inserted into the autonomously replicable vector DNA, which may be introduced into the host so that it is harbored by the host as extrachromosomal DNA as described above. Alternatively, the acid phosphatase gene may be incorporated into chromosome of the host microorganism in accordance with a method which uses transduction, transposon (Biotechnol., 1, 417 (1983)), Mu phage (Japanese Patent Laid-open No. 2-109985), or homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Lab. (1972)).

<5> Expression of acid phosphatase gene by recombinant microorganism

The transformant obtained as described above, into which the recombinant DNA containing the gene coding for the acid phosphatase has been introduced, is capable of expressing the acid phosphatase activity at a high level in its cells by cultivating it in an appropriate medium containing a carbon source, a nitrogen source, inorganic ions, and optionally an organic nutrient source. The carbon source to be adequately used includes, for example, carbohydrates such as glucose, alcohols such as glycerol, and organic acids. The nitrogen source to be used includes, for example, ammonia gas, aqueous ammonia, and ammonium salts. The inorganic ions to be adequately used if necessary include, for example, magnesium ion, phosphate ion, potassium ion, iron ion, and manganese ion. The organic nutrient source to be adequately used includes, for example, vitamins and amino acids as well as those containing them such as yeast extract, peptone, meat extract, corn steep liquor, casein hydrolysate, and soybean hydrolysate. The amount of expression of the acid phosphatase activity may be increased by adding, to the medium, an expression-inducing agent depending on a promoter such as IPTG (isopropyl-β-D-thiogalactopyranoside).

The cultivation condition is also not specifically limited. The cultivation may be performed, for example, under an aerobic condition for about 12 to 48 hours while appropriately controlling pH and temperature within ranges of pH 5 to 8 and temperature of 25 to 40 °C.

After that, microbial cells are harvested from a culture, and a cell-free extract is obtained by disruption, from which the acid phosphatase can be purified. The purification is performed by using an appropriate combination of techniques usually used for enzyme purification such as those described in the aforementioned item <1>. As for the purification, it is not necessarily indispensable to completely purify the acid phosphatase. It is sufficient to achieve removal of

contaminants such as an enzyme which participates in degradation of nucleoside as the substrate.

<6> Production of nucleoside-5'-phosphate ester

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Nucleoside-5'-phosphate ester can be produced in a reaction mixture by allowing the acid phosphatase obtained as described above, and having increased affinity for nucleoside and/or increased temperature stability, such as the mutant acid phosphatase obtained by expressing the gene in a large amount in accordance with the genetic engineering technique as described in the item <5> to make contact and cause the reaction of a nucleoside with a phosphate group donor selected from the group consisting of polyphosphoric acid or a salt thereof, phenylphosphoric acid or a salt thereof, acetylphosphoric acid or a salt thereof, and carbamyl phosphate or a salt thereof. In order to achieve a high productivity in this reaction, it is important to adjust pH of the reaction solution to be weakly acidic in a range of 3.0 to 5.5.

When the gene coding for the acid phosphatase is expressed in a large amount by means of the genetic engineering technique, especially when the gene coding for the mutant acid phosphatase having the increased affinity for the nucleoside is expressed in a large amount, then it is also possible to produce nucleoside-5'-phosphate ester inexpensively and efficiently by using a culture containing microbial cells of the transformant, the microbial cells separated and harvested from the culture, or a product obtained from the microbial cells in accordance with, for example, an immobilizing treatment, an acetone treatment, or a lyophilizing treatment, instead of the purified acid phosphatase.

The nucleoside to be used includes, for example, purine nucleosides such as inosine, guanosine, adenosine, xanthosine, purine riboside, 6-methoxypurine riboside, 2,6-diaminopurine riboside, 6-fluoropurine riboside, 6-thiopurine riboside, 2-amino-6-thiopurine riboside, and mercaptoguanosine; and pyrimidine nucleosides such as uridine, cytidine, 5-aminouridine, 5-hydroxyuridine, 5-bromouridine, and 6-azauridine. As a result of the reaction, these natural type nucleosides and nonnatural type nucleosides are specifically phosphorylated at their 5'-positions, and corresponding nucleoside-5'-phosphate esters are produced respectively.

The nucleoside is desirably added to the reaction solution at a concentration of 1 to 20 g/dl. In the case of use of a nucleoside which is scarcely soluble in water, the reaction yield may be improved by adding boric acid or a surfactant such as dimethyl sulfoxide.

When the nucleoside is produced by fermentation, the fermentation medium after the fermentation as such can be added to the phosphorylation reaction liquid. When an element decomposing the nucleoside-5'-phosphate ester is included in the medium, a purification step is preferably employed so that said element is removed.

As for the phosphate group donor to be used, those usable as the polyphosphoric acid or the salt thereof include, for example, pyrophosphoric acid, tripolyphosphoric acid, trimetaphosphoric acid, tetrametaphosphoric acid, hexametaphosphoric acid, mixtures thereof, sodium salts thereof, potassium salts thereof, and mixtures of these salts. Those usable as the phenylphosphoric acid or the salt thereof include, for example, disodium phenylphosphate, dipotassium phenylphosphate, O,O-diphenylphosphoric acid anhydride, and mixtures thereof. Those usable as the carbamyl phosphate or the salt thereof include, for example, disodium carbamyl phosphate, dipotassium carbamyl phosphate, diammonium carbamyl phosphate, dilithium carbamyl phosphate, and mixtures thereof. Those usable as the acetylphosphoric acid or the salt thereof include, for example, lithium potassium acetylphosphate. The concentration at which the phosphate group donor is used is determined by the concentration of the nucleoside as the phosphate group acceptor. The phosphate group donor is usually used in an amount which is 1 to 5 times that of the nucleoside.

A preferred result is obtained in the reaction usually at a temperature of 20 to 60 °C, preferably 30 to 40 °C at a pH on a weakly acidic side of 3.5 to 6.5, preferably 4.0 to 5.0. A reaction temperature when using the mutant acid phosphatase with an increased temperature stability is 20 to 70 °C, preferably 30 to 60 °C. The reaction may be performed by adopting any one of a stationary method and an agitating method. The reaction time defers depending on the condition such as the activity of the enzyme to be used and the substrate concentration, however, it is 1 to 100 hours.

The nucleoside-5'-phosphate ester thus produced may be collected and separated from a mixture after completion of the reaction by adopting a method to use a synthetic resin for adsorption, a method to use a precipitating agent, and other ordinary methods for collection and separation.

[EXAMPLE]

Embodiments of the present invention will be specifically explained below with reference to Examples, however, the present invention is not limited to these Examples.

The transphosphorylation activity was measured under the following condition using inosine as a substrate. The reaction was performed at pH 5.0 at 30 °C for 10 minutes in a reaction solution (1 ml) containing 40 μ mol/ml of inosine, 100 μ mol/ml of sodium pyrophosphate, 100 μ mol/ml of sodium acetate buffer (pH 5.0), and an enzyme. The reaction was stopped by adding 200 μ l of 2 N hydrochloric acid. After that, precipitates were removed by centrifugation. Then, 5'-Inosinic acid produced by the transphosphorylation reaction was quantitatively measured. An amount of enzyme to

produce 1 µmol of 5'-inosinic acid per 1 minute under this standard reaction condition was defined as 1 unit.

The phosphomonoesterase activity was measured under the following condition using 5'-inosinic acid as a substrate. The reaction was performed at 30 °C for 10 minutes in a reaction solution (1 ml) containing 10 μ mol/ml of 5'-inosinic acid, 100 μ mol/ml of MES/NaOH buffer (pH 6.0), and an enzyme. The reaction was stopped by adding 200 μ l of 2 N hydrochloric acid. After that, precipitates were removed by centrifugation. Then, inosine produced by the hydrolytic reaction was quantitatively measured. An amount of enzyme to produce 1 μ mol of inosine per 1 minute under this standard reaction condition was defined as 1 unit.

Inosine and 5'-inosinic acid were analyzed under the following condition by means of high-performance liquid chromatography (HPLC).

Column:

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Cosmosil 5C18-AR (4.6 x 150 mm) [produced by nacalai tesque];

Mobile phase:

5 mM potassium phosphate buffer (pH 2.8)/methanol = 95/5;

Flow rate:

1.0 ml/min;

Temperature:

room temperature;

15 Detection:

UV 245 nm.

Incidentally, in the reaction to produce nucleoside-5'-phosphate esters using nucleosides other than inosine as raw materials, the nucleosides as raw materials and produced nucleoside-5'-phosphate esters were analyzed by HPLC as described above.

Example 1: Purification and Characterization of Acid Phosphatase Derived from Morganella morganii

A nutrient medium (pH 7.0, 50 ml) containing 1 g/dl of peptone, 0.5 g/dl of yeast extract, and 1 g/dl of sodium chloride was poured into Sakaguchi flasks (500 ml), which was sterilized at 120 °C for 20 minutes. A slant culture of Morganella morganii NCIMB 10466 was inoculated to each of the flasks once with a platinum loop, which was cultivated at 30 °C for 16 hours with shaking. Microbial cells (about 3,000 g), which were harvested from a culture by centrifugation, were suspended in 100 mM potassium phosphate buffer (1 L, pH 7.0). A ultrasonic treatment was performed at 4 °C for 20 minutes to disrupt the microbial cells. The treated suspension was centrifuged to remove its insoluble fraction. Thus a cell-free extract was prepared.

Ammonium sulfate was added to the cell-free extract so that 30 % saturation was achieved. Appeared precipitate was removed by centrifugation, and then ammonium sulfate was further added to supernatant so that 60 % saturation was achieved. Appeared precipitate was collected by centrifugation, and it was dissolved in 100 mM potassium phosphate buffer.

This crude enzyme solution was dialyzed four times against 5 L of 100 mM potassium phosphate buffer (pH 7.0), and then it was applied to a DEAE-Toyopeal 650M column (ϕ 4.1 x 22 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0), followed by washing with 800 ml of 20 mM potassium phosphate buffer (pH 7.0). The transphosphorylation activity was found in a fraction which passed through the column, and thus the fraction was recovered.

The fraction was added with ammonium sulfate so that 35 % saturation was achieved, which was adsorbed to a Butyl-Toyopeal column (\$\phi\$ 3.1 x 26 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing ammonium sulfate at 35 % saturation. Elution was performed by using a linear concentration gradient from 35 % saturation to 20 % saturation in potassium phosphate buffer (pH 7.0).

Active fractions were collected and dialyzed against 1 L of 50 mM potassium phosphate buffer (pH 7.0), followed by being applied to a hydroxyapatite column (φ 5 x 6.5 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0). Elution was performed by using a linear concentration gradient from 50 mM to 300 mM of potassium phosphate buffer (pH 7.0).

Active fractions were collected and concentrated by ultrafiltration. This enzyme solution was applied into a HiLoad TM 16/60 Superdex 200 column (produced by Pharmacia). Elution was performed at a flow rate of 1.0 ml/minute by using 50 mM potassium phosphate buffer containing 100 mM sodium chloride.

In accordance with the procedure as described above, the enzyme exhibiting the transphosphorylation activity was purified from the cell-free extract consequently about 550-fold at a recovery ratio of about 10 %. The specific activity and the recovery ratio in this purification process are shown in Table 1. This enzyme sample was homogeneous on SDS-polyacrylamide gel electrophoresis.

Table 1

Step Recovery	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	ratio (%)
Cell-free extract	597	127,200	0.005	100

Table 1 (continued)

		(**************************************		
Step Recovery	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	ratio (%)
Ammonium sulfate fractionation (30 to 60 %)	568	122,210	0.005	95
3. DEAE-Toyopearl	517	36,498	0.014	87
4. Butyl-Toyopearl	394	1,121	0.351	66
Hydroxyapatite	112	50	2.244	19
6. Superdex 200	63	24	2.030	10

The purified enzyme had the following properties.

- (1) Action: Phosphate group is transferred from a phosphate group donor such as polyphosphoric acid to nucleoside, and nucleoside-5'-phosphate ester is produced. Reversely, this enzyme also exhibits an activity to hydrolyze phosphate ester.
- (2) Substrate specificity: Those which serve as the phosphate group donor in the transphosphorylation reaction include, for example, pyrophosphoric acid, tripolyphosphoric acid, trimetaphosphoric acid, tetrametaphosphoric acid, hexametaphosphoric acid, disodium phenylphosphate, dipotassium phenylphosphate, O,O-diphenylphosphoric acid anhydride, disodium carbamyl phosphate, dipotassium carbamyl phosphate, diammonium carbamyl phosphate, and dilithium carbamyl phosphate. Those which serve as the phosphate group acceptor include, for example, purine riboside, inosine, guanosine, adenosine, xanthosine, uridine, and cytidine. On the other hand, those which undergo the action in the phosphate ester hydrolytic reaction include, for example, inorganic phosphoric acid such as pyrophosphoric acid, tripolyphosphoric acid, trimetaphosphoric acid, tetrametaphosphoric acid, hexametaphosphoric acid; phosphate ester such as disodium phenylphosphate, dipotassium phenylphosphate, O,O-diphenylphosphoric acid anhydride, disodium carbamyl phosphate, dipotassium carbamyl phosphate, diammonium carbamyl phosphate, and dilithium carbamyl phosphate; and 5'-nucleotide such as 5'-purine ribotide, 5'-inosinic acid, 5'-guanylic acid, 5'-adenylic acid, 5'-xanthylic acid, 5'-uridylic acid, and 5'-cytidylic acid.
- (3) Optimum pH: 5.2 (transphosphorylation reaction), 6.5 (phosphate ester hydrolytic reaction).
- (4) pH stability: pH 3.0 to 12.0 (treatment at 30 °C for 60 minutes).
- (5) Optimum temperature: about 35 °C.
- (6) Temperature stability: stable up to 30 °C (treatment at pH 7.0 for 30 minutes).
- (7) Effect of the addition of metal ion and inhibitor: This enzyme exhibits no activation phenomenon relevant to its activity by addition of any metal ion. The activity is inhibited by Ag²⁺, Pb²⁺, Hg²⁺, and Cu²⁺. The activity is also inhibited by iodoacetic acid.
- (8) Molecular weight: A calculated molecular weight is about 190,000 in accordance with high-performance liquid chromatography (TSKgel G-3000SW, produced by Toyo Soda).
- (9) Subunit molecular weight: A calculated subunit molecular weight is about 25,000 in accordance with SDS-polyacrylamide gel electrophoresis.

This enzyme exhibits not only the activity to transfer phosphate group to nucleoside, but also the activity to reversely hydrolyze phosphate ester. Moreover, this enzyme exhibits the phosphate ester hydrolytic activity (phosphomonoestrase activity) which is higher than the transphosphorylation activity by not less than 20 times. Other properties are well coincident with those of a known acid phosphatase produced by a bacterium belonging to the genus Morganella (Microbiology, 140, 1341-1350 (1994)). Accordingly, it has been clarified that this enzyme is an acid phosphatase.

Sodium pyrophosphate (10 g/dl) and inosine (2 g/dl) were dissolved in sodium acetate buffers each having pH of 5.5, 5.0, 4.5, 4.0, and 3.5, to which the enzyme sample described above was added so that a concentration of 50 units/dl was obtained. The reaction mixture was incubated at 30 °C for 6 hours while maintaining each pH, and the amount of produced 5'-inosinic acid was measured along with passage of time. Produced inosinic acid contained only 5'-inosinic acid. By-production of 2'-inosinic acid and 3'-inosinic acid was not observed at all. A result is shown in Fig. 1. The velocity of 5'-inosinic acid production was maximum at pH 5.0. However, the maximum accumulated amount of 5'-inosinic acid was higher at lower pH. The reaction condition at pH 4.0 was most efficient for production of 5'-inosinic acid, in which 5'-inosinic acid was produced and accumulated in an amount of 2.60 g/dl by performing the reaction for 3 hours.

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Example 2: Phosphorylation Reaction of Various Nucleosides by Acid Phosphatase Sample Derived from Morganella morganii

Sodium pyrophosphate (10 g/dl) and inosine, guanosine, uridine, or cytidine (2 g/dl) as a phosphate group acceptor were dissolved in sodium acetate buffer (pH 4.0), to which the enzyme sample prepared in Example 1 was added so that its concentration was 50 units/dl. The reaction mixture was incubated at 30 °C for 3 hours while maintaining pH at 4.0. The amount of nucleoside-5'-ester produced by the reaction is shown in Table 2.

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Produced nucleotide contained only nucleoside-5'-ester. By-production of nucleoside-2'-ester and nucleoside-3'-ester was not observed at all.

Table 2

Nucleoside	Product	Produced amount (g/dl)
Inosine	5'-inosinic acid	2.60
Guanosine	5'-guanylic acid	1.90
Uridine	5'-uridylic acid	1.30
Cytidine	5'-cytidylic acid	0.98

Example 3: Production of 5'-Inosinic acid from Various Phosphate Compounds as Phosphate Group Donors by Acid Phosphatase Sample Derived from Morganella morganii

Inosine (2 g/dl) and sodium tripolyphosphate, sodium polyphosphate (trade name: Polygon P, produced by Chiyoda Chemical), disodium phenylphosphate, or disodium carbamyl phosphate (10 g/dl) as a phosphate group donor were dissolved in sodium acetate buffer (pH 4.0), to which the enzyme sample prepared in Example 1 was added so that its concentration was 50 units/dl. The reaction mixture was incubated at 30 °C for 3 hours while maintaining pH at 4.0. The amount of 5'-inosinic acid produced by the reaction is shown in Table 3.

5'-Inosinic acid was efficiently produced and accumulated by using any of the phosphate group donors. However, the accumulated amount of 5'-inosinic acid was the highest when sodium polyphosphate was used as the phosphate group donor.

Table 3

Phosphate group donor	Produced 5'-inosinic acid (g/dl)		
Sodium tripolyphosphate	2.10		
Sodium polyphosphate	2.72		
Disodium phenylphosphate	2.33		
Disodium carbamyl phosphate	2.54		

Example 4: Purification and Characterization of Acid Phosphatase Derived from Escherichia blattae

A nutrient medium (pH 7.0, 50 ml) containing 1 g/dl of peptone, 0.5 g/dl of yeast extract, and 1 g/dl of sodium chloride was poured into Sakaguchi flasks (500 ml), which was sterilized at 120 °C for 20 minutes. A slant culture of Escherichia blattae JCM 1650 was inoculated to each of the flasks once with a platinum loop, which was cultivated at 30 °C for 16 hours with shaking. Microbial cells were harvested from a culture by centrifugation. The microbial cells (about 3,300 g) were suspended in 100 mM potassium phosphate buffer (1 L, pH 7.0). A ultrasonic treatment was performed at 4 °C for 20 minutes to disrupt the microbial cells. The treated suspension was centrifuged to remove its insoluble fraction. Thus a cell-free extract was prepared.

Ammonium sulfate was added to the cell-free extract so that 30 % saturation was achieved. Appeared precipitate was removed by centrifugation, and then ammonium sulfate was further added to supernatant so that 60 % saturation was achieved. Appeared precipitate was recovered by centrifugation, and it was dissolved in 100 mM potassium phosphate buffer.

This crude enzyme solution was dialyzed four times against 5 L of 100 mM potassium phosphate buffer (pH 7.0), and then it was applied to a DEAE-Toyopeal 650M column (ϕ 6.2 x 35 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0), followed by washing with 20 mM potassium phosphate buffer (pH 7.0). The transphosphorylation activity was found in a fraction which passed through the column, and thus the fraction was collected.

The active fraction was added with ammonium sulfate so that 35 % saturation was achieved, which was applied to a Butyl-Toyopeal column (ϕ 5.0 x 22.5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing

ammonium sulfate at 35 % saturation. Elution was performed by using a linear concentration gradient from 35 % saturation to 20 % saturation in potassium phosphate buffer (pH 7.0).

Active fractions were collected and dialyzed against 1 L of 100 mM potassium phosphate buffer (pH 7.0), followed by being applied to a hydroxyapatite column (ϕ 3.0 x 7.0 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.0). Elution was performed by using a linear concentration gradient from 50 mM to 100 mM of potassium phosphate buffer (pH 7.0), and active fractions were collected.

This enzyme solution was dialyzed against 1 L of 10 mM potassium phosphate buffer (pH 6.0), followed by being applied to a CM-Toyopearl column (ϕ 2.0 x 14.0 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.0). Elution was performed by using a linear concentration gradient in potassium phosphate buffer (pH 6.0) containing from 0 mM to 300 mM potassium chloride. Active fractions eluted from the column were collected.

In accordance with the procedure as described above, the enzyme exhibiting the transphosphorylation activity was purified from the cell-free extract consequently about 600-fold at a recovery ratio of about 16 %. The specific activity and the recovery ratio in this purification process are shown in Table 4. This enzyme sample was homogeneous on SDS-polyacrylamide gel electrophoresis.

Table 4

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Recovery ratio (%)
Cell-free extract	365	160,650	0.002	100
2. Ammonium sulfate fractionation (30 to 60 %)	340	138,895	0.002	93
3. DEAE-Toyopearl	318	30,440	0.010	87
4. Butyl-Toyopearl	232	661	0.347	63
Hydroxyapatite	96	96	1.000	26
6. CM-Toyopearl	59	43	1.365	16

The purified enzyme had the following properties.

- (1) Action: Phosphate group is transferred from a phosphate group donor such as polyphosphoric acid to nucleoside, and nucleoside-5'-phosphate ester is produced. Reversely, this enzyme also exhibits an activity to hydrolyze phosphate ester.
- (2) Substrate specificity: Those which serve as the phosphate group donor in the transphosphorylation reaction include, for example, pyrophosphoric acid, tripolyphosphoric acid, trimetaphosphoric acid, tetrametaphosphoric acid, hexametaphosphoric acid, disodium phenylphosphate, dipotassium phenylphosphate, O,O-diphenylphosphoric acid anhydride, disodium carbamyl phosphate, dipotassium carbamyl phosphate, diammonium carbamyl phosphate, and dilithium carbamyl phosphate. Those which serve as the phosphate group acceptor include, for example, purine riboside, inosine, guanosine, adenosine, xanthosine, uridine, and cycidine. On the other hand, those which undergo the action in the phosphate ester hydrolytic reaction include, for example, inorganic phosphoric acid such as pyrophosphoric acid, tripolyphosphoric acid, trimetaphosphoric acid, tetrametaphosphoric acid, hexametaphosphoric acid; phosphate ester such as disodium phenylphosphate, dipotassium phenylphosphate, O,O-diphenylphosphoric acid anhydride, disodium carbamyl phosphate, dipotassium carbamyl phosphate, diammonium carbamyl phosphate, and dilithium carbamyl phosphate; and 5'-nucleotide such as 5'-purine ribotide, 5'-inosinic acid, 5'-guanylic acid, 5'-adenylic acid, 5'-xanthylic acid, 5'-uridylic acid, and 5'-cytidylic acid.
- (3) Optimum pH: 5.2 (transphosphorylation reaction), 6.5 (phosphate ester hydrolytic reaction).
- (4) pH stability: pH 3.5 to 12.0 (treatment at 30 °C for 60 minutes).
- (5) Optimum temperature: about 35 °C.
- (6) Temperature stability: stable up to 40 °C (treatment at pH 7.0 for 30 minutes).
- (7) Effect of the addition of metal ion and inhibitor: This enzyme exhibits no activation phenomenon relevant to its activity by addition of any metal ion The activity is inhibited by Fe²⁺, Ag²⁺, Pb²⁺, Hg²⁺, and Cu²⁺. The activity is also inhibited by iodoacetic acid.
 - (8) Molecular weight: A calculated molecular weight is about 188,000 in accordance with high-performance liquid chromatography (TSKgel G-3000SW, produced by Toyo Soda).
- (9) Subunit molecular weight: A calculated subunit molecular weight is about 24,500 in accordance with SDS-polyacrylamide gel electrophoresis.

This enzyme also exhibits not only the activity to transfer phosphate group to nucleoside, but also the activity to

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reversely hydrolyze phosphate ester, in the same manner as the enzyme purified from the cell-free extract of <u>Morganella morganii</u> NCIMB 10466. Moreover, this enzyme exhibits the phosphate ester hydrolytic activity (phosphomonoesterase activity) which is higher than the transphosphorylation activity by not less than 30 times. Accordingly, it has been clarified that this enzyme is an acid phosphatase.

Sodium pyrophosphate (15 g/dl) and inosine (3 g/dl) were dissolved in sodium acetate buffers each having pH of 5.5, 5.0, 4.5, 4.0, and 3.5, to which the enzyme sample described above was added so that a concentration of 50 units/dl was obtained. The reaction mixture was incubated at 30 °C for 6 hours while maintaining each pH, and the amount of produced 5'-inosinic acid was measured along with passage of time. Produced inosinic acid contained only 5'-inosinic acid. By-production of 2'-inosinic acid and 3'-inosinic acid was not observed at all. A result is shown in Fig. 2. The velocity of 5'-inosinic acid production was maximum at pH 5.0. However, the maximum accumulated amount of 5'-inosinic acid was higher at lower pH. The reaction condition at pH 4.0 was most efficient for production of 5'-inosinic acid. 5'-Inosinic acid was produced and accumulated in an amount of 1.56 g/dl by performing the reaction at 30 °C at pH 4.0 for 3 hours.

Example 5: Phosphorylation Reaction of Various Nucleosides by Acid Phosphatase Sample Derived from Escherichia blattae

Sodium pyrophosphate (15 g/dl) and inosine, guanosine, uridine, or cytidine (3 g/dl) were dissolved in sodium acetate buffer (pH 4.0), to which the enzyme sample prepared in Example 4 was added so that its concentration was 50 units/dl. The reaction mixture was incubated at 35 °C for 3 hours while maintaining pH at 4.0. The amount of produced nucleoside-5'-ester is shown in Table 5.

Produced nucleotide contained only nucleoside-5'-ester. By-production of nucleoside-2'-ester and nucleoside-3'-ester was not observed at all.

Table 5

Nucleoside	Product	Produced amount (g/dl)
Inosine	5'-inosinic acid	1.56
Guanosine	5'-guanylic acid	1.05
Uridine	5'-uridylic acid	1.87
Cytidine	5'-cytidylic acid	1.22

Example 6: Production of 5'-Inosinic acid from Various Phosphate Compounds as Phosphate Group Donors by Acid Phosphatase Sample Derived from Escherichia blattae

Inosine (2 g/dl) and sodium tripolyphosphate, sodium polyphosphate (trade name: Polygon P, produced by Chiyoda Chemical), disodium phenylphosphate, or disodium carbamyl phosphate (10 g/dl) as a phosphate group donor were dissolved in sodium acetate buffer (pH 4.0), to which the enzyme sample prepared in Example 4 was added so that its concentration was 50 units/dl. The reaction miture was incubated at 35 °C for 3 hours while maintaining pH at 4.0. The amount of produced 5'-inosinic acid is shown in Table 6.

5'-Inosinic acid was efficiently produced and accumulated by using any of the phosphate group donors. However, the accumulated amount of 5'-inosinic acid was the highest when sodium polyphosphate was used as the phosphate group donor.

Table 6

Phosphate group donor	Produced 5'-inosinic acid (g/dl)
Sodium tripolyphosphate	1.20
Sodium polyphosphate	1.79
Disodium phenylphosphate	1.50
Disodium carbamyl phosphate	1.53

Example 7: Isolation of Gene Coding for Acid Phosphatase from Chromosome of Morganella morganii

(1) Determination of N-terminal amino acid sequence

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The acid phosphatase purified from the cell-free extract of Morganella morganii NCIMB 10466 in accordance with

the method described in Example 1 was adsorbed to DITC membrane (produced by Milligen/Biosearch), and its N-terminal amino acid sequence was determined by using Prosequencer 6625 (produced by Milligen/Biosearch). An N-terminal amino acid sequence comprising 20 residues shown in SEQ ID NO: 1 in Sequence Listing was determined.

(2) Isolation of DNA fragment containing gene coding for acid phosphatase

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Chromosomal DNA was extracted from cultivated microbial cells of Morganella morganii NCIMB 10466 in accordance with a method of Murray and Thomson (Nucl. Acid Res., 4321, 8 (1980)). The chromosomal DNA was partially degraded with restriction enzyme Sau3Al. After that, DNA fragments of 3 to 6 kbp were fractionated by means of sucrose density gradient centrifugation. A plasmid vector pUC118 (produced by Takara Shuzo) was digested with restriction enzyme BamHI, which was ligated with the partially degraded chromosomal DNA fragments. DNA ligation was performed by using DNA ligation kit (produced by Takara Shuzo) in accordance with a designated method. After that, Escherichia coli JM109 (produced by Takara Shuzo) was transformed with an obtained DNA mixture in accordance with an ordinary method. Transformants were plated on an L agar medium containing 100 µg/ml of ampicillin, and they were grown to prepare a gene library.

A reaction solution containing 4 mM p-nitrophenylphosphoric acid and 100 mM MES/NaOH buffer (pH 6.5) was poured onto a surface of the agar medium on which the transformants had grown, and the temperature was kept at 30 °C for 15 minutes. Strains which had expressed the phosphatase activity liberated p-nitrophenol and exhibited a yellow color. Accordingly, transformants were selected by using this phenomenon as an index. As a result of screening for a gene expression library comprising about 20,000 strains of transformants, 30 strains of transformants which had expressed the phosphatase activity were obtained.

The transformants (30 strains), which had expressed the phosphatase activity, were subjected to single colony isolation. Single colonies were inoculated to an L-medium (2.5 ml) containing 100 μg/ml of ampicillin, and they were cultivated at 37 °C for 16 hours. Sodium acetate buffer (100 mM, pH 5.0, 50 μl) containing inosine (2 g/dl) and sodium pyrophosphate (10 g/dl) was added to microbial cells harvested from culture, and the reaction mixture was incubated at 30 °C for 16 hours. Production of 5'-inosinic acid was detected by HPLC analysis to select microbial strains having the transphosphorylation activity. As a result, we succeeded in obtaining 5 strains of transformants which exhibited the transphosphorylation activity and which were assumed to harbor a DNA fragment containing the objective acid phosphatase gene.

Example 8: Determination of Nucleotide Sequence of Acid Phosphatase Gene Derived from Morganella morganii NCIMB 10466

The inserted DNA fragment was analyzed by preparing a plasmid in accordance with an alkaline lysis method (Molecular Cloning 2nd edition (J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbour Laboratory Press, pl. 25 (1989)) from one strain of the transformants which were assumed to harbor the DNA fragment containing the acid phosphatase gene derived from Morganella morganii NCIMB 10466 obtained in Example 7. This plasmid was designated as pMPI501. Fig. 3 shows a determined restriction enzyme map of the inserted DNA fragment.

The region of the acid phosphatase gene was further specified by subcloning. As a result, it was suggested that this acid phosphatase gene was contained in a fragment having a size of 1.2 Kbp excised by restriction enzymes HindIII and EcoRI. Thus in order to determine the nucleotide sequence, plasmid DNA was constructed in which the fragment of 1.2 kbp was ligated with pUC118 having been digested with HindIII and EcoRI. Escherichia coli JM109 (produced by Takara Shuzo) was transformed with this plasmid DNA designated as pMPI505 in accordance with an ordinary method, which was plated on an L agar medium containing 100 µg/ml of ampicillin to obtain a transformant.

The plasmid was extracted in accordance with the alkaline lysis method from the transformant of Escherichia coli JM109 (produced by Takara Shuzo) harboring pMPI505 to determine the nucleotide sequence. The nucleotide sequence was determined in accordance with a method of Sanger (J. Mol. Biol., 143, 161 (1980)) by using Taq DyeDeoxy Terminator Cycle Sequencing Kit (produced by Applied Biochemical). A nucleotide sequence of a determined open reading frame is shown in SEQ ID NO: 2 in Sequence Listing. An amino acid sequence of the protein deduced from the nucleotide sequence is shown in SEQ ID NO: 3 in Sequence Listing. A partial sequence, which was completely coincident with the N-terminal amino acid sequence of the purified enzyme, was found in the amino acid sequence. The N-terminal of the purified enzyme starts from the 21th alanine residue of the sequence shown in SEQ ID NO: 3. Accordingly, it is assumed that the amino acid sequence shown in SEQ ID NO: 3 is that of a precursor protein, and that a peptide comprising a range from the 1st methionine residue to the 20th alanine residue is eliminated after translation. An amino acid sequence of a mature protein thus deduced is shown in SEQ ID NO: 4 in Sequence Listing. A molecular weight of the mature protein estimated from the amino acid sequence is calculated to be 24.9 kilodaltons, which is well coincident with the result of SDS-PAGE for the purified enzyme. According to the results described above, and because of the fact that the transformant harboring the plasmid containing this fragment exhibited the transphos-

phorylation activity, it was identified that this open reading frame was the region coding for the objective acid phosphatase.

The nucleotide sequence and the amino acid sequence were respectively compared with known sequences for homology. Data bases of EMBL and SWISS-PROT were used. As a result, it has been revealed that the nucleotide sequence shown in SEQ ID NO: 2 in Sequence Listing is coincident with a nucleotide sequence of a known acid phosphatase gene derived from Morganella morganii (Thaller, M. C. et al., Microbiology, 140, 1341 (1994)) except that 54th G is A, 72th G is A, 276th T is G, 378th T is C, 420th G is T, 525th C is G, 529th C is T, and 531th G is A in the latter, and that the amino acid sequence shown in SEQ ID NO: 4 in Sequence Listing is the same as that of the acid phosphatase gene derived from Morganella morganii. Namely, the gene, which codes for the protein comprising the amino acid sequence shown in SEQ ID NO: 4 in Sequence Listing, is the acid phosphatase gene of Morganella morganii NCIMB 10466.

A precursor protein comprises 249 amino acids, and a molecular weight of the protein deduced from its sequence is 27.0 kilodaltons.

The strain of Escherichia coli JM109 transformed by a plasmid pMPI505, has been designated as AJ13143, which has been internationally deposited on February 23, 1996 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) under the provision of the Budapest Treaty, and awarded a deposition number of FERM BP-5422.

Example 9: Amplification of Activity by Expressing Gene of Acid Phosphatase Derived from Morganella morganii NCIMB 10466

Escherichia coli JM109/pMPI505 constructed in Example 8 was inoculated to an L-medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and it was cultivated at 37 °C for 16 hours. Microbial cells were harvested from its culture by centrifugation, and they were washed once with physiological saline. The microbial cells were suspended in 100 mM potassium phosphate buffer (5 ml, pH 7.2), and they were disrupted by means of a ultrasonic treatment performed at 4 °C for 20 minutes. The treated solution was centrifuged to remove an insoluble fraction, and thus, a cell-free extract was prepared.

The transphosphorylation activity of the obtained cell-free extract was measured while using controls of cell-free extracts prepared from the wild type strain of Morganella morganii and Escherichia coli JM109 transformed with the plasmid pUC118 in the same manner as described above. A result is shown in Table 7. The transphosphorylation activity was not detected in Escherichia coli JM109/pUC118. The transphosphorylation activity was also low in the wild type strain of Morganella morganii. On the other hand, Escherichia coli JM109/pMPI505 exhibited a high transphosphorylation activity which was 150 times as high as that of the wild type strain of Morganella morganii in specific activity. According to the result, it has been demonstrated that the introduced DNA fragment allows Escherichia coli to express the acid phosphatase at a high level.

Table 7

Microbial strain	Transphosphorylation Activity (units/mg)
Morganella morganii NCIMB 10466	0.008
Escherichia coli JM109/pUC118	not detected
Escherichia coli JM109/pMPI505	1.250

Example 11: Isolation of Gene Coding for Acid Phosphatase from Chromosome of Escherichia blattae

(1) Determination of N-terminal amino acid sequence

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The acid phosphatase purified from the cell-free extract of <u>Escherichia blattae</u> JCM 1650 was adsorbed to DITC membrane (produced by Milligen/Biosearch), and its N-terminal amino acid sequence was determined by using Prosequencer 6625 (produced by Milligen/Biosearch). An N-terminal amino acid sequence comprising 15 residues shown in SEQ ID NO: 8 in Sequence Listing was determined.

(2) Isolation of DNA fragment containing gene coding for acid phosphatase

Chromosomal DNA was extracted from cultivated cells of <u>Escherichia blattae</u> JCM 1650 in accordance with a method of Murray and Thomson (Nucl. Acid Res., 4321, 8 (1980)). The chromosomal DNA was partially degraded with <u>Sau</u>3Al. After that, DNA fragments of 3 to 6 kbp were fractionated by means of sucrose density gradient centrifugation.

A plasmid vector pUC118 (produced by Takara Shuzo) was digested with <u>Bam</u>HI, which was ligated with the partially degraded chromosomal DNA fragments. DNA ligation was performed by using DNA ligation kit (produced by Takara Shuzo) in accordance with a designated method. After that, <u>Escherichia coli JM109</u> (produced by Takara Shuzo) was transformed with an obtained DNA mixture in accordance with an ordinary method. Transformants were plated on an L agar medium containing 100 µg/ml of ampicillin, and they were grown to prepare a gene library.

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A reaction solution containing 4 mM p-nitrophenylphosphoric acid and 100 mM MES/NaOH buffer (pH 6.5) was poured onto a surface of the agar medium on which the transformants had grown, and the temperature was kept at 30 °C for 15 minutes. Strains which had expressed the phosphatase activity liberated p-nitrophenol and exhibited a yellow color. Accordingly, transformants were selected by using this phenomenon as an index. As a result of screening for a chromosomal gene expression library comprising about 8,000 strains of transformants, 14 strains of transformants which had expressed the phosphatase activity were obtained.

The transformants (14 strains), which had expressed the phosphatase activity, were subjected to single colony isolation. Single colonies were inoculated to an L-medium (2.5 ml) containing 100 µg/ml of ampicillin, and they were cultivated at 37 °C for 16 hours. Sodium acetate buffer (100 mM, pH 5.0, 50 µl) containing inosine (2 g/dl) and sodium pyrophosphate (10 g/dl) was added to microbial cells harvested from culture liquids to perform the reaction at 30 °C for 16 hours. Production of 5'-inosinic acid was detected by HPLC analysis to select strains having the transphosphorylation activity. As a result, we succeeded in obtaining 3 strains of transformants which exhibited the transphosphorylation activity and which were assumed to harbor a DNA fragment containing the objective acid phosphatase gene.

Example 12: Determination of Nucleotide Sequence of Acid Phosphatase Gene Derived from Escherichia blattae JCM 1650

The inserted DNA fragment was analyzed by extracting a plasmid in accordance with the alkaline lysis method from one strain of the transformants which were assumed to harbor the DNA fragment containing the acid phosphatase gene derived from Escherichia blattae JCM 1650 obtained in Example 11. This plasmid was designated as pEPI301. Fig. 5 shows a determined restriction enzyme map of the inserted DNA fragment.

The region of the acid phosphatase gene was further specified by subcloning. As a result, it was suggested that this acid phosphatase gene was included in a fragment having a size of 2.4 Kbp excised by restriction enzymes <u>Clal</u> and <u>Bam</u>HI. Thus in order to determine the nucleotide sequence, plasmid DNA was constructed in which the fragment was ligated with pBluescript KS(+) (produced by Stratagene) having been digested with <u>Clal</u> and <u>Bam</u>HI. <u>Escherichia coli</u> JM109 (produced by Takara Shuzo) was transformed with the plasmid DNA designated as pEPI305 in accordance with an ordinary method, which was plated on an L agar medium containing 100 μg/ml of ampicillin to obtain a transformant.

The plasmid was extracted in accordance with the alkaline lysis method from the transformant of Escherichia coli JM109 (produced by Takara Shuzo) harboring pEPI305 to determine the nucleotide sequence. A nucleotide sequence of a determined open reading frame is shown in SEQ ID NO: 6 in Sequence Listing. An amino acid sequence of the protein deduced from the nucleotide sequence is shown in SEQ ID NO: 7 in Sequence Listing. A partial sequence, which was completely coincident with the N-terminal amino acid sequence of the purified enzyme, was found in the amino acid sequence. The N-terminal of the purified enzyme starts from the 19th leucine residue of the sequence shown in SEQ ID NO: 7. Accordingly, it is assumed that the amino acid sequence shown in SEQ ID NO: 7 is that of a precursor protein and that a peptide comprising a range from the 1st methionine residue to the 18th alanine residue is eliminated after translation. An amino acid sequence of a mature protein thus deduced is shown in SEQ ID NO: 8 in Sequence Listing. Accordingly, an estimated molecular weight of the mature protein is calculated to be 25.1 kilodaltons, which is well coincident with the result of SDS-PAGE for the purified enzyme. According to the results described above, and because of the fact that the transformant harboring the plasmid containing this fragment exhibited the transphosphorylation activity, it was identified that this open reading frame was the region coding for the objective acid phosphatase.

Namely, the gene, which codes for the protein comprising the amino acid sequence shown in SEQ ID NO: 8 in Sequence Listing, is the acid phosphatase gene of <u>Escherichia blattae JCM.1650</u>.

The nucleotide sequence and the amino acid sequence were respectively compared with known sequences for homology. Data bases of EMBL and SWISS-PROT were used. As a result, it has been revealed that the protein shown in SEQ ID NO: 8 and DNA coding for it are novel. A precursor protein encoded by this gene comprises 249 amino acids, and a molecular weight of the protein deduced from its sequence is 27.0 kilodaltons.

The amino acid sequence was compared with known sequences respectively for homology. As a result, this protein exhibited a high degree of homology with the acid phosphatase of <u>Providencia stuartii</u> (77.1 %) with the acid phosphatase of <u>Morganella morganii</u> in Example 8 (77.1 %), and with acid phosphatase of <u>Salmonella typhimurium</u> (44.3 %).

The strain of Escherichia coli JM109 transformed by a plasmid pEPI305, has been designated as AJ13144, which has been internationally deposited on February 23, 1996 in National Institute of Bioscience and Human Technology of

Agency of Industrial Science and Technology (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) under the provision of the Budapest Treaty, and awarded a deposition number of FERM BP-5423.

Example 13: Amplification of Activity by Expressing Gene of Acid Phosphatase Derived from Escherichia blattae JCM 1650

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Escherichia coli JM109/pEPI305 constructed in Example 12 was inoculated to an L medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and it was cultivated at 37 °C for 16 hours. Microbial cells were harvested from its culture by centrifugation, and they were washed once with physiological saline. The microbial cells were suspended in 100 mM potassium phosphate buffer (5 ml, pH 7.2), and were disrupted by means of a ultrasonic treatment performed at 4 °C for 20 minutes. The treated solution was centrifuged to remove an insoluble fraction, and thus a cell-free extract was prepared.

The transphosphorylation activity of the obtained cell-free extract was measured while using controls of cell-free extracts prepared from the wild type strain of Escherichia coli JM109 transformed with the plasmid pBluescript KS(+) in the same manner as described above. A result is shown in Table 8. The transphosphorylation activity was not detected in Escherichia coli JM109/pBluescript KS(+). The transphosphorylation activity was also low in the wild type strain of Escherichia coli 'M109/pEP1305 exhibited a high transphosphorylation activity which was 120 times as high as that of the wild type strain of Escherichia blattae in sepcific activity. According to the result, it has been demonstrated that the introduced DNA fragment allows Escherichia coli to express the acid phosphatase at a high level.

Table 8

Microbial strain	Transphosphorylation Activity (units/mg)
Escherichia blattae JCM 1650 Escherichia coli JM109/pBluescript KS(+) Escherichia coli JM109/pEPI305	0.002 not detected 0.264

Example 14; Production of 5'-Inosinic Acid from Inosine by Using Strain Harboring Acid Phosphatase Gene Derived from Escherichia blattae JCM 1650

Sodium pyrophosphate (12 g/dl) and inosine (6 g/dl) were dissolved in 100 mM sodium acetate buffer (pH 4.0), to which the microbial cells of Escherichia coli JM109/pEPI305 described above were added to give a cell concentration of 200 mg/dl as converted into a dry weight of the microbial cells. The reaction mixture was incubated at 35 °C for 10 hours while maintaining pH at 4.0, and the amount of produced 5'-inosinic acid was measured along with passage of time. Produced inosinic acid contained only 5'-inosinic acid. By-production of 2'-inosinic acid and 3'-inosinic acid was not observed at all. A result is shown in Fig. 6. 5'-Inosinic acid was produced and accumulated extremely efficiently in a short period of time in the reaction to produce 5'-inosinic acid from pyrophosphate and inosine by using this microorganism.

Example 15: Preparation of A Gene Encoding An Acid Phosphatase with lowered Phosphomonoesterase acitivity

As described in Examples 13 and 14, the strain harboring the acid phosphatase gene derived from Escherichia blattae expresses a considerable amount of the acid phosphatase, and 5'-inosinic acid is produced and accumulated extremely efficiently in a short period of time in the reaction to produce 5'-inosinic acid from pyrophosphate and inosine by using this microorganism. However, it has been revealed that the accumulated amount of 5'-inosinic acid does not exceed a certain degree because produced 5'-inosinic acid undergoes degradation by the phosphomonoesterase activity possessed by the acid phosphatase itself. Thus the enzyme was intended to be improved by introducing mutation into the acid phosphatase gene derived from Escherichia blattae cloned in Example 11, in accordance with the site-directed mutagenesis method by using PCR.

Oligonucleotides MUT300, MUT310, and MUT320 shown in SEQ ID NOs. 9, 10, and 11 in Sequence Listing were synthesized respectively in accordance with the phosphoamidite method by using a DNA synthesizer (Model 394 produced by Applied Biosystems).

The plasmid pEPI305 (1 ng) as a template prepared in Example 12, M13 primer RV (produced by Takara Shuzo) and MUT310 oligonucleotide (each 2.5 μmol) as primers, and Taq DNA polymerase (2.5 units, produced by Takara Shuzo) were added to 100 mM Tris-HCl buffer (pH 8.3, 100 μl) containing dATP, dCTP, dGTP, dTTP (each 200 μM), potassium chloride (50 mM), and magnesium chloride (1.5 mM) to perform a PCR reaction in which a cycle comprising

periods of 30 seconds at 94 °C, 2 minutes at 55 °C, and 3 minutes at 72 °C was repeated 25 times. The PCR reaction was performed by using Thermal Cycler PJ2000 type (produced by Takara Shuzo). Also, a PCR reaction was performed in the same manner as described above by using plasmid pEPI305 (1 ng) as a temperate, and M13 primer M3 (produced by Takara Shuzo) and MUT300 oligonucleotide (each 2.5 µmol) as primers. Each of the reaction solutions was purified by gel filtration to remove the primers by using Micro spin column S-400 (produced by Pharmacia).

Each of the PCR reaction products (1 μl) was added to 100 mM Tris-HCl buffer (pH 8.3, 95 μl) containing dATP, dCTP, dGTP, dTTP (each 200 μM), potassium chloride (50 mM), and magnesium chloride (1.5 mM), and it was heated at 94 °C for 10 minutes, followed by cooling to 37 °C over 60 minutes. After that, the temperature was kept at 37 °C for 15 minutes to form a heteroduplex. Taq DNA polymerase (2.5 units) was added thereto to perform a reaction at 72 °C for 3 minutes so that the heteroduplex was completed. After that, M13 primer RV and M13 primer M3 (each 2.5 μmol) were added to this reaction solution to perform a PCR reaction in which a cycle comprising periods of 30 seconds at 94 °C, 2 minutes at 55 °C, and 3 minutes at 72 °C was repeated 10 times.

A product of the second PCR reaction was digested with <u>Clal</u> and <u>BamHI</u> followed by phenol/chloroform extraction and ethanol precipitation. This DNA fragment was ligated with pBluescript KS(+) having been digested with <u>Clal</u> and <u>BamHI</u>. <u>Escherichia coli</u> JM109 (produced by Takara Shuzo) was transformed with obtained plasmid DNA in accordance with an ordinary method, which was plated on an L agar medium containing 100 µg/mI of ampicillin to obtain a transformant.

The plasmid was extracted from the transformant in accordance with the alkaline lysis method to determine its nucleotide sequence, confirming that the objective nucleotide was substituted. Thus a mutant gene coding for a mutant phosphatase was prepared in which the 74th glycine residue (GGG) of the mature protein was substituted with an aspartic acid residue (G*A*T). The plasmid containing this mutant gene was designated as pEPI310.

A mutant gene coding for a mutant phosphatase was prepared in which the 153th isoleucine residue (ATC) of the mature protein was substituted with a threonine residue (A*CC), in accordance with the same procedure as described above by using pEPI305 as a template, and MUT300 and MUT320 oligonucleotides as primers. The plasmid containing this mutant gene was designated as pEPI320. Further, a mutant gene coding for a mutant phosphatase was prepared in which the 74th glycine residue (GGG) of the mature protein was substituted with an aspartic acid residue (G*A*T), and the 153th isoleucine residue (ATC) of the mature protein was substituted with a threonine residue (A*CC), in accordance with the same procedure as described above by using pEPI310 as a template, and MUT300 and MUT320 oligonucleotides as primers. The plasmid containing this mutant gene was designated as pEPI330.

Escherichia coli JM109/pEPI310, Escherichia coli JM109/pEPI320, and Escherichia coli JM109/pEPI330 into which the plasmids containing the respective mutant acid phosphatase genes had been introduced, and Escherichia coli JM109/pEPI305 into which the plasmid containing the wild type acid phosphatase gene had been introduced were inoculated to an L medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and they were cultivated at 37 °C for 16 hours. Microbial cells were harvested from their culture, and they were washed once with physiological saline. The microbial cells were suspended in 100 mM potassium phosphate buffer (5 ml, pH 7.0), and they were disrupted by means of a ultrasonic treatment performed at 4 °C for 20 minutes. The treated solutions were centrifuged to remove insoluble fractions, and thus cell-free extracts were prepared. Phosphomonoesterase activities and transphosphorylation acitivities of the obtained cell-free extracts were measured at pH 4.0, and they were compared with an activity of the wild strain.

Table 9 shows the result of measurement of phosphomonoesterase activities and transphosphorylation acitivities of wild type acid phosphatase and acid phosphatases with lowered phosphomonoesterase activity. It shows that both of phosphomonoesterase activities and transphosphorylation acitivities of acid phosphatases with lowered phosphomonoesterase activity are lowered as compared with wild type acid phosphatase, and that degrees of decrease of phosphomonoesterase activities are larger than that of transphosphorylation activities, with the result that a ratio of phosphomonoesterase activity to transphosphorylation activity of the mutant acid phosphatase is lowered in comparison with the wild type acid phosphatase.

Table 9

Plasmid	Phosphomonoesterase activity (units/mg)	Transphosphorylation activity (units/mg)	Ratio 1) (Relative value)
pEPI305	2.38	0.132	18.03 (100)

1): Ratio of phosphomonoesterase activities to the activities to produce nucleoside-5'-phosphate ester

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Table 9 (continued)

Plasmid	Phosphomonoesterase activity (units/mg)	Transphosphorylation activity (units/mg)	Ratio 1) (Relative value)
pEPI310	0.26	0.019	13.68(76)
pEPI320	0.88	0.123	7.15(39)
pEPI330	0.42	0.070	6.00(33)

^{1):} Ratio of phosphomonoesterase activities to the activities to produce nucleoside-5'-phosphate ester

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Example 16: Production of 5'-Inosinic Acid from Inosine by Using The Strains Horboring A Gene Encoding The Acid Phosphatase with Lowered Phosphomonoesterase Activity

Escherichia coli JM109/pEPI310, Escherichia coli JM109/pEPI320, and Escherichia coli JM109/pEPI330 into which the plasmids containing the genes encoding the acid phosphatases with lowered phosphomonoesterase activity had been introduced, and Escherichia coli JM109/pEPI305 into which the plasmid containing the wild type acid phosphatase gene had been introduced were inoculated to an L medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and they were cultivated at 37 °C for 16 hours.

Sodium pyrophosphate (12 g/dl) and inosine (6 g/dl) were dissolved in sodium acetate buffer (pH 4.0), to which microbial cells of each of the strains of Escherichia coli obtained by the cultivation described above were added to give a cell concentration of 200 mg/dl as converted into a dry weight of the microbial cells. The reaction mixture was incubated at 35 °C for 32 hours while maintaining pH at 4.0, and the amount of produced 5'-inosinic acid was measured along with passage of time. A result is shown in Fig. 8.

In Fig. 7, the axis of ordinate indicates the concentration of 5'-inosinic acid (mg/dl), and the axis of abscissa indicates the reaction time (h). Progress of the reaction is indicated by solid circles for <u>Escherichia coli</u> JM109/pEPI305, solid triangles for <u>Escherichia coli</u> JM109/pEPI310, blanked circles for <u>Escherichia coli</u> JM109/pEPI320, and blanked squares for <u>Escherichia coli</u> JM109/pEPI330, as measured by using the cells of the respective strains.

The velocity of degradation of produced 5'-inosinic acid was decreased in the reaction to produce 5'-inosinic acid from inosine by using the stains harboring the acid phosphatase with lowered phosphomonoesterase activity. As a result, the yield and the accumulated amount of 5'-inosinic acid were increased. The highest accumulation of 5'-inosinic acid was exhibited by Escherichia coli JM109/pEPI330 as the strain harboring the gene encoding the acid phosphatase with lowered phosphomonoesterase activity in which the 74th glycine residue and the 153th isoleucine residue were substituted with the aspartic acid residue and the threonine residue respectively.

Example 17: Production of Various Nucleoside-5'-Phosphate Esters by Using The Strains Harboring A Gene Encoding The Acid Phosphatase with Lowered Phosphomonoesterase Activity

Escherichia coli JM109/pEPI330 into which the plasmid containing the gene encoding the acid phosphatase with lowered phosphomonoesterase activity had been introduced was inoculated to an L medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and it was cultivated at 37 °C for 16 hours.

Sodium pyrophosphate (12 g/dl), and inosine, guanosine, uridine, or cytidine (6 g/dl) as a phosphate group acceptor were dissolved in 100 mM sodium acetate buffer (pH 4.0), to which the microbial cells described above were added to give a cell concentration of 200 mg/dl as converted into a dry weight of the cells. The reaction mixture was incubated at 35 °C for 32 hours while maintaining pH at 4.0. Amounts of produced nucleoside-5'-phosphate esters are shown in Table 10. Produced nucleotide contained only nucleoside-5'-phosphate ester. By-production of nucleoside-2'-phosphate ester and nucleoside-3'-phosphate ester was not observed at all.

Table 10

Nucleoside	Product	Produced amount (g/dl)
Inosine	5'-inosinic acid	7.45
Guanosine	5'-guanylic acid	4.77
Uridine	5'-uridylic acid	8.93
Cytidine	5'-cytidylic acid	6.60

Example 18: Production of 5'-Inosinic Acid from Various Phosphate Compounds as Phosphate Group Donors by Using

The Straing Harboring A Gene Encoding The Acid Phosphatase with Lowered Phosphomonoesterase Activity

Escherichia coli JM109/pEPI330 into which the plasmid containing the mutant acid phosphatase gene had been introduced was inoculated to an L medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and it was cultivated at 37 °C for 16 hours.

Inosine (6 g/dl) and sodium tripolyphosphate, sodium polyphosphate (trade name: Polygon P, produced by Chiyoda Chemical), disodium phenylphosphate, or disodium carbamyl phosphate (12 g/dl) as a phosphate group donor were dissolved in 100 mM sodium acetate buffer (pH 4.0), to which the microbial cells described above were added to give a cell concentration of 200 mg/dl as converted into a dry weight of the cells. The reaction mixture was incubated at 35 °C for 32 hours while maintaining pH at 4.0. The amount of produced 5'-inosinic acid is shown in Table 11. 5'-Inosinic acid was efficiently produced and accumulated by using any of the phosphate group donors. However, the accumulated amount of 5'-inosinic acid was the highest when polyphosphoric acid was used as the phosphate group donor.

Table 11

Phosphate group donor	Produced 5'-inosinic acid (g/dl)
Sodium tripolyphosphate	5.96
Sodium polyphosphate	8.84
Disodium phenylphosphate	7.60
Disodium carbamyl phosphate	7.73

Example 19

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Studies on production of a new mutant acid phosphatase gene derived from E. blattae JCM1650 and enzymological properties of the mutant acid phosphatase gene:

In Examples 19 to 22, the transphosphorylation activity to a nucleoside was measured under the following conditions. The reaction was conducted at 30°C and a pH of 4.0 for 10 minutes using 1 ml of a reaction solution containing 40 µmol/ml of inosine, 100 µmol/ml of sodium pyrophosphate, 100 µmol/ml of a sodium acetate buffer (pH 4.0) and an enzyme. This reaction was terminated with the addition of 200 µl of 2-N hydrochloric acid. Then, the precipitate was removed through centrifugation, and the amount of 5'-inosinic acid formed through the transphosphorylation was determined under the above-mentioned conditions. The amount of the enzyme with which to produce 1 µmol of inosinic acid under these standard reaction conditions was defined as 1 unit.

Further, the transphosphorylation activity was measured by changing the inosine concentration from 10 to 100 µmol/ml under the reaction conditions of the above-mentioned composition, and the rate constant of inosine in the transphosphorylation activity was determined using the Hanes-Woolf plot [Biochem. J., 26, 1406 (1932)].

As described later, the detailed analysis was conducted with respect to the mutant enzyme by which to improve the productivity of the nucleoside-5'-phosphate ester described in Example 15. Consequently, it was found that the affinity for nucleoside of the mutant enzyme was improved by 2 times as compared with that of the wild-type enzyme. Therefore, the present inventors considered that the productivity of the nucleoside-5'-phosphate ester would be improved by increasing the affinity for nucleoside of the above-mentioned enzyme, and they further modified the enzyme by the genetic engineering method.

Plasmid pEPI305 containing the gene encoding the wild-type acid phosphatase derived from E. blattae described in Example 15 was used, and the site specific mutation was introduced into this plasmid DNA by the genetic engineering method to produce a gene encoding the mutant acid phosphatase. pEPI305 is a plasmid DNA formed by binding a DNA fragment of 2.4 Kbp cleaved with restriction endonucleases Clal and BamHI and containing a gene encoding a wild-type acid phosphatase derived from E. blattae JCM1650 to pBluescript KS(+) (supplied by Stratagene) cleaved with Clal and BamHI. The base sequence of the gene encoding the acid phosphatase is represented by SEQ ID NO: 6 in Sequence Listing. Further, an amino acid sequence of a precursor protein anticipated from this base sequence is represented by SEQ ID NO: 7 in Sequence Listing. From the analytical results of the purified enzyme (described in Example 4), the amino acid sequence of the maturation protein is presumed to be represented by SEQ ID NO: 8 in Sequence Listing.

Oligonucleotides MUT300 (SEQ ID NO: 9 in Sequence Listing), MUT310 (SEQ ID NO: 10 in Sequence Listing), MUT320 (SEQ ID NO: 11 in Sequence Listing), MUT330 (SEQ ID NO: 12 in Sequence Listing), MUT340 (SEQ ID NO: 13 in Sequence Listing), MUT350 (SEQ ID NO: 14 in Sequence Listing), MUT360 (SEQ ID NO: 15 in Sequence Listing), MUT370 (SEQ ID NO: 16 in Sequence Listing), MUT380 (SEQ ID NO: 17 in Sequence Listing) and MUT390 (SEQ ID NO: 18 in Sequence Listing) having the sequences shown in Sequence Listing were synthesized by the phosphoamidite method using a DNA synthesizer (Model 394 supplied by Applied Biosystem).

One nanogram of pEPI305 as a template, 2.5 µmols of M13 Primer RV (supplied by Takara Shuzo Co., Ltd.), 2.5

μmols of oligonucleotide MUT310 and 2.5 units of tac DNA polymerase (supplied by Takara Shuzo Co., Ltd.) were added to 100 μl of a Tris-hydrochloride buffer (pH 8.3) containing 200 μM of dATP, 200 μM of dCTP, 200 μM of dGTP, 200 μM of dTTP, 50 mM of potassium chloride and 1.5 mM of magnesium chloride. PCR was conducted in which a three-part step, namely, at 94°C for 30 seconds, at 55°C for 2 minutes and at 72°C for 3 minutes was repeated 25 times. In this reaction, a thermal cycler PJ2000 model (supplied by Takara Shuzo Co., Ltd.) was used. Separately, PCR was likewise conducted using 1 ng of plasmid DNA pEPI305 as a template, 2.5 μmols of M13 Primer M3 (supplied by Takara Shuzo Co., Ltd.) as a primer and 2.5 μmols of oligonucleotide MUT300. Each of the reaction solutions was purified through gel filtration using a microspin column S-400 (supplied by Pharmacia) to remove the primer.

One microliter of each of the PCR solutions was added to 95 μ l of a 100-mM Tris-hydrochloride buffer (pH 8.3) containing 200 μ M of dATP, 200 μ M of dCTP, 200 μ M of dGTP, 200 μ M of dTTP, 50 mM of potassium chloride and 1.5 mM of magnesium chloride. The mixture was heated at 94°C for 10 minutes, then cooled to 37°C over the course of 60 minutes, and warmed at 37°C for 15 minutes to form a heteroduplex. To this were £Jded 2.5 units of tac DNA polymerase, and the reaction was conducted at 72°C for 3 minutes to complete the heteroduplex. Subsequently, 2.5 μ mols of M13 Primer RV and 2.5 μ mols of M13 Primer M3 were added to the reaction solution, and PCR was conducted in which a three-part step, namely, at 94°C for 30 seconds, at 55°C for 2 minutes and at 72°C for 3 minutes was repeated 10 times.

The second PCR product was cleaved with Clal and BamHI, then extracted with a mixture of phenol and chloroform, and precipitated with ethanol. This DNA fragment was bound to pBluescript KS (+) cleaved with Clal and BamHI. E. coli JM109 (supplied by Takara Shuzo Co., Ltd.) was transformed in a usual manner using the resulting plasmid DNA. This was plated on an L agar medium containing 100 μg/mI of ampicillin to obtain a transformant. A plasmid was prepared from the transformant by an alkali bacteriolysis method, the base sequence was determined, and it was identified that the desired base was substituted. The determination of the base sequence was conducted by the method of Sanger et al. [J. Mol. Biol., 143, 161 (1980)] using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (supplied by Applied Biochemical). In this manner, the mutant gene encoding the mutant phosphatase in which the 74th glycine residue (GGG) of the maturation protein was substituted with the aspartic acid residue (G*A*T) was produced. This mutant gene-containing plasmid was designated pEPI310 (Example 15).

The above-mentioned procedure was repeated using the plasmid having the mutation introduced therein as a template to cumulatively introduce the site-specific mutation. A plasmid was produced from the transformant by the alkali bacteriolysis method, the base sequence was determined, and it was identified that the desired base was substituted. The resulting mutant genes encoding the mutant phosphatase and the mutation sites are shown in Table 12. The amino acid residue in the mutation site indicates an amino acid residue in the amino acid sequence of the mature protein represented by SEQ ID NO: 8 in Sequence Listing.

Table 12

plasmid name	starting material	primer	mutation position and substituted amino acid
pEPI305	-		wild type
pEPI310	pEPI305	MUT300	74Gly(GGG)→ASp(G*A*T)
		MUT310	
pEPI330	pEPI310	MUT300 MUT320	74Gly(GGG)→Asp(G*A*T) 153lle(ATC)→Thr(A*CC)
pEPI340	pEPI330	MUT300 MUT330	63Leu(CTG)→Gln(C*AG) 65Ala(GCG)→Gln(*C*AG) 66Glu(GAA)→Ala(G*CA) 74Gly(GGG)→Asp(G*A*T) 153lle(ATC)→Thr(A*CC)
pEPI350	pEPI340	MUT300 MUT340	63Leu(CTG)→Gln(C*AG) 65Ala(GCG)→Gln(*C*AG) 66Glu(GAA)→Ala(G*CA) 74Gly(GGG)→Asp(G*A*T) 85Ser(TCC)→Tvr(T*AC) 153lle(ATC)→Thr(A*CC)
pEPI360	pEPI340	MUT300 MUT350	63Leu(CTG)→Gln(C*AG) 65Ala(GCG)→Gln(*C*AG)

Table 12 (continued)

	plasmid name	starting material	primer	mutation position and substituted amino acid
		-		66Glu(GAA)→Ala(G*CA)
				74Gly(GGG)→Asp(G*A*T)
				135Thr(ACC)→Lys(A*A*A)
	•			136Glu(GAG)→Asp(GA*C)
:				153lle(ATC)→(A*CC)
	pEPI370	pEPI360	MUT300	63Leu(CTG)→Gln(C*AG)
			MUT360	65Ala(GCG)→Gln(*C*AG)
				66Glu(GAA)→Ala(G*CA)
				69Asn(AAC)→Asp(*GAC)
				71Ser(AGC)→Ala(*G*CC)
		,		72Ser(AGT)→Ala(*G*CT)
				74Gly(GGG)→Asp(G*A*T)
				135Thr(ACC)→Lys(A*A*A)
				136Glu(GAG)→Asp(GA*C)
				153lle(ATC)→(A*CC)
	pEPI380	pEPI370	MUT300	63Leu(CTG)→Gln(C*AG)
	•	•	MUT370	65Ala(GCG)→Gln(*C*AG)
				66Glu(GAA)→Ala(G*CA)
	,			69Asn(AAC)→Asp(*GAC)
				71Ser(AGC)→Ala(*G*CC)
				72Ser(AGT)→Ala(*G*CT)
				74Gly(GGG)→Asp(G*A*T)
	1			116Asp(GAT)→Glu(GA*A)
				135Thr(ACC)→Lys(A*A*A)
				136Glu(GAG)→Asp(GA*C)
				153lle(ATC)→(A*CC)
·	pEPI390	pEPI380	MUT300	63Leu(CTG)→Gln(C*AG)
ł			MUT380	65Ala(GCG)→Gln(*C*AG)
			-	66Glu(GAA)→Ala(G*CA)
				69Asn(AAC)→Asp(*GAC)
				71Ser(AGG)→Ala(*G*CC)
j		·		72Ser(AGT)→Ala(*G*CT)
				74Gly(GGG)→Asp(G*A*T)
- 1				116Asp(GAT)→Glu(GA*A)
J				130Ser(TCT)→Glu(*G*A*A)
				135Thr(ACC)→Lys(A*A*A)
				136Gln(GAG)→Asp(GA*C)
				153lle(ATC)→Thr(A*CC)
	pEPI400	pEP1380	MUT300	63Leu(CTG)→Gln(C*AG)
			MUT390	65Ala(GCG)→Gln(*C*AG)
				66Glu(GAA)→Ala(G*CA)
				69Asn(AAC)→Asp(*GAC)
- {				71Ser(AGC)→Ala(*G*CC)
				72Ser(AGT)→AIN(G*A*T)
		,		74Gly(GGG)→Asp(G*A*T)
				92Ala(GCC)→Ser(*A*GC)
				94Ala(GCg)→Glu(G*A*A)
				116Asp(GAT)→Glu(GA*A)
				, , , , , , , , , , , , , , , , , , ,

Table 12 (continued)

plasmid name	starting material	primer	mutation position and substituted amino acid
			135Thr(ACC)→Lys(A*A*)
			136Glu(GAG)→(GA*C)
			153lle(ATC)→Thr(A*CC)

Each of E. coli JM109/pEPI330, E. coli JM109/pEPI340, E. coli JM109/pEPI350, E. coli JM109/pEPI360, E. coli JM109/pEPI370, E. coli JM109/pEPI380, E. coli JM109/pEPI390 and E. coli JM109/pEPI400 each having introduced therein a plasmid containing the mutant acid phosphatase gene and E. coli JM109/pEPI305 having introduced therein a plasmid containing a wild-type acid phosphatase gene was inoculated into 50 ml of an L medium containing 100 μg/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours. The cells were collected from 2 liters of the culture solution of each of the strains through centrifugation, and washed once with a physiological saline solution. The cells were suspended in 50 ml of a 100-mM phosphate buffer (pH 7.0), and sonicated at 4°C for 20 minutes to disrupt the cells. The thus-treated solution was centrifuged to remove insoluble fractions and prepare a cell-free extract. Each of the acid phosphatases was purified from each of the cell-free extracts by the method described in Example 4. Each of the enzyme products was uniform in the SDS-polyacrylamide electrophoresis.

The rate constant of inosine in the transphosphorylation of the purified mutant acid phosphatases and wild-type acid phosphatase was measured, and the results are shown in Table 13. It was found that the mutant enzyme expressed in E. coli JM109/pEPI330 having the improved productivity of the nucleoside-5'-phosphate ester as described in Example 15 has decreased Vmax but greatly decreased the Km value to inosine which means the increased affinity for inosine by twice or more as compared with the wild-type enzyme expressed in E. coli JM109/pEPI305. This suggested that the productivity of the nucleoside-5'-phosphate ester of this mutant enzyme was greatly improved not only because of the decrease in the nucleotidase activity but also because of the improvement in the affinity for nucleoside which was an important factor. Accordingly, it was expected that the increase in the affinity for nucleoside leads to the improvement in the productivity.

The new mutant enzymes expressed in E. coli JM109 having been introduced therein with the new mutant enzyme gene produced in this Example exhibited the affinity for inosine which was more improved than that of E. coli JM109/pEPI330 described in Example 15. Thus, it was expected that the productivity of the nucleoside-5'-phosphate ester was improved. Further, the mutant enzyme expressed in E. coli JM109/pEPI380 not only improved the affinity for inosine but also increased the Vmax value as compared with the wild-type enzyme. Still further, it was expected that the productivity of the nucleoside-5'-phosphate ester was improved.

Table 13

Strain of an enzyme	Km(mM)	Vmax(unit/mg)
E. coli JM109/pEPI305	202	1.83
E. coli JM109/pEPI330	109	1.39
E. coli JM109/pEPI340	85	1.03
E. coli JM109/pEPI350	85	0.93
E. coli JM109/pEPI360	55	1.33
E. coli JM109/pEPI370	42	1.15
E. coli JM109/pEPI380	42	2.60
E. coli JM109/pEPI390	42	2.58
E. coli JM109/pEPI400	43	2.11

Example 20

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Production of 5'-inosinic acid using a new mutant acid phosphatase gene-containing strain:

Each of E. coli JM109/pEPI330, E. coli JM109/pEPI340, E. coli JM109/pEPI360, E. coli JM109/pEPI370 and E. coli JM109/pEPI380 each having introduced therein the plasmid containing the mutant acid phosphatase gene was inoculated into 50 ml of an L medium containing 100 μg/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours.

Pyrophosphoric acid (15 g/dl) and 8 g/dl of inosine were dissolved in an acetate buffer (pH 4.0). To the solution was added E. coli JM109 strain having introduced therein the above-mentioned mutant and wild-type acid phosphatase genes such that the concentration reached 200 mg/dl in terms of the dry cell weight. The reaction was conducted at

35°C for 32 hours while maintaining the pH at 4.0, and the amount of 5'-inosinic acid formed over the course of time was measured. Inosinic acid formed was only 5'-inosinic acid, and the formation of 2'-inosinic acid and 3'-inosinic acid as by-products was not observed at all. The results are shown in Fig. 8.

E. coli JM109/pEPI330 described in Example 15 showed the accumulation of 5'-inosinic acid in a large amount. Although the substrate still remained, the formation of 5'-inosinic acid stopped when the amount of 5'-inosinic acid accumulated reached 7.5 g/dl, and the amount of 5'-inosinic acid was no longer increased. By contrast, the new mutant acid phosphatase gene-containing strains provided the large amount of 5'-inosinic acid accumulated. Especially, in the reaction using E. coli JM109/pEPI370 and E. coli JM109/pEPI380, the larger amount of 5'-inosinic acid accumulated was provided. In addition, the reaction rate was high, showing that the productivity of 5'-inosinic acid was further improved greatly. In particular, in E. coli JM109/pEPI380, the reaction rate was high, and quite a high reactivity was shown.

Example 21

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Production of various nucleoside-5'-phosphate esters using a new mutant acid phosphatase gene-containing strain;

E. coli JM109/pEPI380 having introduced therein the plasmid containing the new mutant acid phosphatase gene was inoculated into 50 ml of an L medium containing 100 μ g/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours.

Pyrophosphoric acid (15 g/dl) and 8 g/dl of inosine, guanine, uridine or cytidine as a phosphate acceptor were dissolved in a 100-mM acetate buffer (pH 4.5). To this was added the above-mentioned strain such that the concentration reached 100 mg/dl in terms of the dry cell weight. The reaction was conducted at 35°C for 12 hours while maintaining the pH at 4.0. The amount of the nucleoside-5'-phosphate ester formed is shown in Table 4. The phosphorylation proceeded well with any of the nucleosides to form and accumulate the corresponding nucleoside-5'-phosphate esters. The nucleotide formed was only the nucleoside-5'-phosphate ester, and the formation of a nucleoside-2'-phosphate ester and a nucleoside-3'-phosphate ester as by-products was not observed at all.

Table 14

Nucleoside	Product	Amount of the Product (g/dl)
inosine	5'-inosinic acid	12.05
guanosine	5'-guanylic acid	5.78
uridine	5'-uridylic acid	13.28
cytidine	5'-cytidylic acid	10.65

Example 22

<u>Production of 5'-inosinic acid using a new acid phosphatase gene-containing strain and various phosphoric acid compounds as a phosphate donor:</u>

E. coli JM109/pEPI380 having introduced therein the plasmid containing the new mutant acid phosphatase gene was inoculated into 50 ml of an L medium containing 100 μg/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours.

Inosine (6 g/dl) and 15 g/dl of a tripolyphosphate, a polyphosphate ("Polygon P", a trade name for a product of Chiyoda Kagaku K.K.), disodium phenylacetate or disodium carbamylphosphate were dissolved in a 100-mM acetate buffer (pH 4.0). To this was added the above-mentioned strain such that the concentration reached 100 mg/dl in terms of the dry cell weight. The reaction was conducted at 35°C for 12 hours while maintaining the pH at 4.0. The amount of 5'-inosinic acid formed was shown in Table 15. 5'-Inosinic acid was formed and accumulated at good efficiency with any of the phosphate donors. Especially when a polyphosphate was used as a phosphate donor, 5'-inosinic acid was accumulated in the largest amount.

Table 15

	7.2.2.2
Phosphate donor	Amount of 5'-inosinic acid formed (g/dl)
sodium tripolyphosphate	10.84
sodium polyphosphate	13.35
disodium phenylphosphate	12.84
disodium carbamylphosphate	12.42

Table 15 (continued)

Phosphate donor	Amount of 5'-inosinic acid formed (g/dl)
potassium lithium acetylphosphate	10.65

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Example 23: Isolation of Acid Phosphatase Gene Derived from Chromosome of Providencia stuartii and Determination of Nucleotide Sequence of the Gene

Oligonucleotides, PRP1 and PRP2, having nucleotide sequences illustrated in SEQ ID NO: 19 and 20 in Sequence Listing, respectively, were synthesized. These oligonucleotides are designed to amplify a gene coding for acid phosphatase of Providencia stuartii on the basis of known nucleotide sequence of the gene coding for acid phosphatase of Providencia stuartii (Database of EMBL Accession number X64820).

Chromosomal DNA was extracted from cultivated microbial cells of <u>Providencia stuartii</u> ATCC 29851 in accordance with a method of Murray and Thomson (Nucl. Acid Res., 4321, 8 (1980)). The chromosomal DNA (0.1 ng) as a template, oligonucleotides PRP1 and PRP2 (each 2.5 μmol) as primers, and Taq DNA polymerase (2.5 units, produced by Takara Shuzo) were added to 100 mM Tris-HCl buffer (pH 8.3, 100 μl) containing dATP, dCTP, dGTP, dTTP (each 200 μM), potassium chloride (50 mM), and magnesium chloride (1.5 mM) to perform a PCR reaction in which a cycle comprising periods of 30 seconds at 94 °C, 2 minutes at 55 °C, and 3 minutes at 72 °C was repeated 30 times. The reaction solution was subjected to agarose gel electrophoresis, followed by recovering the amplified DNA fragment of about 1 kbp'by means of glass powders (made by Takara Shuzo). The gene fragment was digested with <u>Bam</u>HI, which was ligated with pUC118 degiested with <u>Bam</u>HI. The plasmid obtained as described above was designated as pPRP100.

Phosphomonoesterase activity and transphosphorylation activity of <u>Escherichia coli</u> JM109/pPRP100, a transformant to which pPRP100 was introduced, were mesured. As a result, the strain showed an activity to transphosphorylate to nucleoside as well as phosphomonoesterase activity.

The plasmid was extracted in accordance with the alkaline lysis method from the transformant of <u>Escherichia coli</u> JM109/pPRP100 to determine the nucleotide sequence. A nucleotide sequence of a determined open reading frame and an amino acid sequence of the protein deduced from the nucleotide sequence are shown in SEQ ID NO: 21 and 22 in Sequence Listing. The nucleotide sequence of the open reading frame is completely coincident with the nucleotide sequence of the known acid phosphatase gene of <u>Providencia stuartii</u>.

Example 24: Isolation of Acid Phosphatase Gene Derived from Chromosomes of Enterobacter aerogenes, Klebsiella planticola and Serratia ficaria and Determination of Nucleotide Sequences of the Genes

Chromosomal DNA was extracted from cultivated microbial cells of Enterobacter aerogenes IFO 12010, Klebsiella planticola IFO 14939 and Serratia ficaria IAM 13540 in accordance with a method of Murray and Thomson (Nucl. Acid Res., 4321, 8 (1980)). Then, in accordance with the method described in Example 7(2), a chromosomal gene expression library comprising about 20,000 transformants of Escherichia coli JM109 was constructed and screened to obtain transformants which showed transphosphorylation activity. It was considered that each of these transformants harbours the acid phosphatase gene derived from each of the original strains.

Plasmid DNA was extracted from one of the transformants of <u>Escherichia coli</u> which was considered to have the acid phosphatase gene derived from <u>Enterobacter aerogenes</u> IFO 12010 in accordance with an alkaline lysis method and the inserted DNA of the plasmid was analyzed. The above plasmid was designated as pENP100. A restriction enzyme map of the inserted DNA derived from <u>Enterobacter aerogenes</u> IFO 12010 is shown in Fig. 9.

As a result of specifying the region of acid phosphatase gene by subcloning, it was suggested that the acid phosphatase gene is contained in the 1.6 kbp fragment excised by restriction enzymes <u>Sall</u> and <u>Kpnl</u>. Then, the <u>Sall-Kpnl</u> fragment was ligated with pUC118 which was digested with <u>Sall</u> and <u>Kpnl</u> to construct a plasmid. The resulting plasmid was designated as pENP110.

According to the procedure as described above, plasmid DNA was extracted from one of the transformants of Escherichia coli which was considered to have the acid phosphatase gene derived from Klebsiella planticola IFO 14939 in accordance with an alkaline lysis method and the insert DNA of the plasmid was analyzed. The above plasmid was designated as pKLP100. A restriction enzyme map of the inserted DNA derived from Klebsiella planticola IFO 14939 is shown in Fig. 10.

As a result of specifying the region of acid phosphatase gene by subcloning, it was suggested that the acid phosphatase gene is contained in the 2.2 kbp fragment excised by restriction enzymes <u>KpnI</u> and <u>EcoRI</u>. Then, the <u>KpnI-EcoRI</u> fragment was ligated with pUC118 which was digested with <u>KpnI</u> and <u>EcoRI</u> to construct a plasmid. The resulting plasmid was designated as pKLP110.

Similarly, plasmid DNA was extracted from one of the transformants of Escherichia coli which was considered to

have the acid phosphatase gene derived from <u>Serratia ficaria</u> IAM 13540 in accordance with an alkaline lysis method and the inserted DNA of the plasmid was analyzed. The above plasmid was designated as pSEP100. A restriction enzyme map of the inserted DNA derived from <u>Serratia ficaria</u> IAM 13540 is shown in Fig. 11.

As a result of specifying the region of acid phosphatase gene by subcloning, it was suggested that the acid phosphatase gene is contained in the 1.4 kbp fragment excised by restriction enzymes <u>Hind</u>III. Then, the <u>Hind</u>III fragment was ligated with pUC118 which was digested with <u>Hind</u>III to construct a plasmid. The resulting plasmid was designated as pSEP110.

Then, the plasmid DNAs were extracted from the transformants, <u>Escherichia coli JM109/pENP110</u>, <u>Escherichia coli JM109/pENP110</u> and <u>Escherichia coli JM109/pSEP110</u>, to which pENP110 pKLP110 and pSEP110 had been introduced, respectively, in accordance with an alkaline lysis method. The nucleotide sequences of inserts of these plasmids were determined in accordance with the method described in Example 8. The determined nucleotide sequences of open reading frames of the inserts are shown in SEQ ID NO:23 for <u>Enterobacter aerogenes</u> IFO 12010, in SEQ ID NO: 25 for <u>Klebsiella planticola</u> IFO 14939 and in SEQ ID NO: 27 for <u>Serratia ficaria IAM 13540</u>. Additionally, the deduced amino acid sequences are shown in SEQ ID NOs: 24, 26 and 28, respectively. Because of the fact that the transformants harboring the plasmids containing these fragments exhibited the transphosphorylation activity, it was identified that these open reading frames were the objective acid phosphatase genes.

The nucleotide sequences and the deduced amino acid sequences were respectively compared with known sequences for homology. Data bases of EMBL and SWISS-PROT were used. As a result, it has been revealed that the genes illustrated in SEQ ID NO: 23, 25 and 27 in Sequence Listing are new genes. It is assumed that the protein encoded by the gene derived from Enterobacter aerogenes IFO 12010 comprises 248 amino acid residues, the protein encoded by the gene derived from Klebsiella planticola IFO 14939 comprises 248 amino acid residues and the protein encoded by the gene derived from Serratia ficaria IAM 13540 comprises 244 amino acid residues. There is a possibility that these proteins may be precursor proteins like the acid phosphatases derived from Morganella morganii and Escherichia blattae.

The amino acid sequences deduced from the nucleotide sequences are shown in Fig. 12 in one-letter together with the deduced amino acid sequence of the acid phosphatase derived from Morganella morganii NCIMB 10466 obtained in Example 8, that of Escherichia blattae JCM 1650 obtained in Example 12 and the known amino acid sequence of the acid phosphatase of Providencia stuartii (EMBL Accession number X64820). Common amino acid residues among all of the amino acids sequences are indicated with asterisks under the sequences in Fig. 12.

As shown in Fig. 12, the amino acid sequences of the acid phosphatases derived from six strains are highly homologous each other and 130 amino acid residues are common among all of the amino acid sequences. Thus, it is assumed that these acid phosphatases have similar functions.

Example 25: Amplification of Activity by Expressing Gene of Acid Phosphatase Derived from Enterobacter aerogenes, Klebsiella planticola and Serratia ficaria

Escherichia coli JM109/pPRP100 constructed in Example 23, Escherichia coli JM109/pENP110, Escherichia coli JM109/pKLP110 and Escherichia coli JM109/pSEP110 constructed in Example 24 were inoculated to an L-medium (50 ml) containing 100 μg/ml of ampicillin and 1 mM of IPTG, and were cultivated at 37 °C for 16 hours. Microbial cells were harvested from these cultures by centrifugation, and they were washed once with physiological saline. The microbial cells were suspended in 100 mM potassium phosphate buffer (5 ml, pH 7.0), and they were disrupted by means of a ultrasonic treatment performed at 4 °C for 20 minutes. The treated solutions were centrifuged to remove an insoluble fraction, and thus cell-free extracts were prepared.

The transphosphorylation activities of the obtained cell-free extracts were measured while using controls of cell-free extracts prepared from Providencia stuartii ATCC 29851, Enterobacter aerogenes IFO 12010, Klebsiella planticola IFO 14939, Serratia ficaria IAM 13450, and Escherichia coli JM109 transformed with the plasmid pUC118 in the same manner as described above. Results are shown in Table 16. The transphosphorylation activities were low in all of the wild type strains. The transphosphorylation activity was not detected in Escherichia coli JM109/pUC118. On the other hand, the transformants of Escherichia coli JM109 to which the acid phosphatase genes were introduced exhibited high transphosphorylation activities in comparison with wild type strains. According to the result, it has been demonstrated that each of the introduced DNA fragment allow Escherichia coli to express the acid phosphatase at a high level.

Table 16

Microbial strain	Transphosphrylation Activity (units/mg)
Providencia stuartii ATCC 29851	0.005
Enterobacter aerogenes IFO 12010	0.002

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Table 16 (continued)

Microbial strain	Transphosphrylation Activity (units/mg)
Klebsiella planticola IFO 14939	0.002
Serratia ficaria IAM 13450	0.001
Escherichia coli JM109/pUC118	not detected
Escherichia coli JM109/pPRP100	0.833
Escherichia coli JM109/pENP110	0.301
Escherichia coli JM109/pKLP110	0.253
Escherichia coli JM109/pSEP110	0.123

Example 26

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Production of a mutant acid phosphatase gene having an improved temperature stability:

As described in Examples 20, 21 and 22, the E. blattae-derived mutant acid phosphatase gene-containing strain produced in Example 19 expressed the considerable amount of the acid phosphatase. In the production of 5'-inosinic acid from pyrophosphoric acid and inosine using this strain, 5'-inosinic acid was formed and accumulated in the high conversion yield. The optimum reaction temperature of this acid phosphatase was 35°C. However, when this reaction was conducted at a higher temperature, the reaction rate was increased, and the reaction was conducted upon increasing the nucleoside concentration of the phosphate acceptor in the reaction solution. Accordingly, it was expected that the nucleoside-5'-phosphate ester could be produced more efficiently for a shorter period of time. Thus, the temperature stability of the enzyme was improved upon introducing the mutation into the E. blattae-derived acid phosphatase gene cloned in Example 19 by the site specific mutation method using PCR.

Plasmid pEPI380 containing the gene encoding the E. blattae JCM1650-derived mutant acid phosphatase described in Example 19 was used, and the site specific mutation was introduced into this plasmid DNA by the genetic engineering method to produce the gene encoding the mutant acid phosphatase having the increased temperature stability. pEPI380 is a plasmid DNA obtained by binding a DNA fragment of 2.4 Kbp containing the gene encoding the mutant acid phosphatase derived from E. blattae JCM1650 and cleaved with restriction endonucleases C1al and BamHI to pBluescript KS(+) (supplied by Stratagene) cleaved with C1al and BamHI. The amino acid sequence of the mature protein anticipated from the base sequence of the gene encoding the acid phosphatase is presumed to be 11 amino acid resides shown in Table 12 in Example 19 in the sequence represented by SEQ ID NO: 8 in Sequence Listing.

Oligonucleotides MUT300 (SEQ ID NO: 9 in Sequence Listing), MUT400 (SEQ ID NO: 29 in Sequence Listing) and MUT410 (SEQ ID NO: 30 in Sequence Listing) having the sequences shown in Sequence Table were synthesized by the phosphoamidite method using a DNA synthesizer (Model 394 supplied by Applied Biosystem).

A mutant gene encoding a mutant phosphatase in which the 104th glutamic acid residue (GAG) of a maturation protein was substituted with a glycine residue (GG*T*) was produced by the method with PCR as in Example 15 using pEPI380 described in Example 19 as a template and MUT300 and MUT410 as primers for introduction of mutation. This mutant gene-containing plasmid was designated pEPI410. Likewise, a mutant gene encoding a mutant phosphatase in which the 151st threonine residue (ACC) was substituted with an alanine residue (G*CC) was produced using pEPI380 as a template and oligonucleotides MUT300, MUT310 and MUT420 as primers for introduction of mutation. This mutant gene-containing plasmid was designated pEPI420.

A plasmid was produced from the transformant of E. coli JM109 having introduced therein plasmids pEPI410 and pEPI420 containing the mutant phosphatase gene by the alkali bacteriolysis method, the base sequence was determined, and it was identified that the desired base was substituted.

Each of E. coli JM109/pEPI410 and E. coli JM109/pEPI420 having introduced therein the mutant acid phosphatase gene as produced in this Example and E. coli JM109/pEPI380 described in Example 19 was inoculated in 50 ml of an L medium containing 100 μg/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours. The cells were collected from 50 ml of the culture solution of each of the strains, and washed once with a physiological saline solution. These cells were suspended in 5 ml of a 100-mM phosphate buffer (pH 7.0), and sonicated at 4°C for 20 minutes to mill the cells. The thus-treated solution was centrifuged to remove insoluble fractions and prepare a cell-free extract.

The cell-free extract formed from each of the strains was warmed at temperatures ranging from 0°C to 80°C with a pH of 7.0 for 30 minutes. After the completion of the warming, the transphosphorylation was conducted under the standard reaction conditions of pH of 4.0 and 30°C using the cell-free extracts treated at various temperatures, and the residual activity was measured. The results are shown in Fig. 13. The mutant enzyme expressed in E. coli JM109/pEPI380 described in Example 19 was stable in the treatment at 40°C for 30 minutes, but the decrease in the activity was observed at higher temperatures. By contrast, the new mutant enzyme expressed in E. coli JM109/pEPI410

and E. coli JM109/pEPI420 having introduced therein the new mutant enzyme gene as produced in this Example improved the temperature stability, and the decrease in the activity was not observed even through the treatment at 50°C for 30 minutes. It was thus expected that when a nucleoside-5'-phosphate ester was produced using these strains at a high temperature, the productivity was further improved.

Example 27

<u>Production of 5'-inosinic acid and 5'-guanylic acid using a mutant acid phosphatase gene-containing strain having an improved temperature stability:</u>

Each of E. coli JM109/pEPI410 and E. coli JM109/pEPI420 having been introduced therein with the mutant acid phosphatase gene and E. coli JM109/pEPI380 described in Example 19 was inoculated in 50 ml of an L medium containing 100 μg/ml of ampicillin and 1 mM of IPTG, and incubated at 37°C for 16 hours.

Pyrophosphoric acid (15 g/dl) and 8 g/dl of inosine or guanosine were dissolved in an acetate buffer (pH 4.0). To this was added E. coli JM109 strain having introduced therein each mutant acid phosphatase gene such that the concentration reached 100 mg/dl in terms of the dry cell weight. The reaction was conducted at 50°C for 9 hours while maintaining the pH at 4.0, and the amount of 5'-inosinic acid or 5'-guanylic acid formed was measured. The results are shown in Table 17. The nucleoside phosphate ester formed was only a nucleoside-5'-phosphate ester, and the production of a nucleoside-2'-phosphate ester and a nucleoside-3'-phosphate ester as by-products was not observed at all. The reaction was also conducted at 35°C for 12 hours using E. coli JM109/pEPI380 strain as a control. The results are also shown in Table 17.

As described in Example 21, the nucleoside-5'-phosphate ester was formed and accumulated efficiently with E. coli JM109/pEPI380. By contrast, when the reaction was conducted using E. coli JM109/pEPI410 and E. coli JM109/pEPI420 having been introduced therein with the new mutant acid phosphatase gene derived from E. blattae as produced in Example 26, 5'-inosinic acid or 5'-guanylic acid in the same amount was formed and accumulated for a shorter period of time. Thus, the nucleoside-5'-phosphate ester could be produced more efficiently. Especially when using E. coli JM109/pEPI420, not only was the reaction time shortened, but also were 5'-inosinic acid and 5'-guanylic acid accumulated in larger amounts, and quite a high productivity was shown.

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Table	

Strain	Reaction	Reaction	Amount of	Amount of
	(°C)	rime (nr)	5'-1hosinc acid formed (g/dl)	5'-Inosinc acid formed 5'-guanylic acid formed (g/dl)
E. coli JM109/pEPI380	30	12	12.05	5.78
E. coli JM109/pEP1410	50	σ	11.85	5.80
E. coli JM109/pEIP420	50	6	12.60	6.11

Annex to the description

	SEQUENCE LISTING	
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	INFORMATION FOR SEQ ID NO:1:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 20 amino acids	
	(B) TYPE: amino acid	
10	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: peptide	
	(v) PROTEIN FRAGMENT TYPE: N-termianl	
	(vi) ORIGINAL SOURCE:	
	(A) ORGANISM: Morganella morganii	
15	(B) STRAIN: NCIMB 10466	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:	
	Ala Ile Pro Ala Gly Asn Asp Ala Thr Thr Lys Pro Asp Leu Tyr Tyr	
	1 5 10 15	
	Leu Lys Asn Glu	
20	20	
	·	
-	INFORMATION FOR SEQ ID NO:2:	
	(i) SEQUENCE CHARACTERISTICS:	
25	(A) LENGTH: 750 base pairs	
25	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: genomic DNA	
30	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	
	(vi) ORIGINAL SOURCE:	
	(A) ORGANISM: Morganella morganii	
	(B) STRAIN: NCIMB 10466	
<i>35</i>	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 1747	
	(ix) FEATURE:	
	(A) NAME/KEY: sig_peptide	
40	(B) LOCATION: 160	
	(ix) FEATURE:	
	(A) NAME/KEY:mat_peptide	
	(B) LOCATION: 61747	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	4.0
45	ATG AAG AAG AAT ATT ATC GCC GGT TGT CTG TTC TCA CTG TTT TCC CTT	48
	Met Lys Lys Asn Ile Ile Ala Gly Cys Leu Phe Ser Leu Phe Ser Leu	
	-20 -15 -10 -5	0.6
	TCC GCG CTG GCC GCG ATC CCG GCG GGC AAC GAT GCC ACC ACC AAG CCG	96
	Ser Ala Leu Ala Ala Ile Pro Ala Gly Asn Asp Ala Thr Thr Lys Pro	
50	ב דט בעם ביד הבד דבד הבה בה ביד ביד הבד הבד הבד הבד הבד הבד הבד הבד הבד הב	1 4 4

			15			Lys		20					25		_		
	TTA	CCG	CCA	CCG	CCG	GAA	GTC	GGC	AGT	ATT	CAG	TTT	TTA	AAT	GAŤ	CAG	192
5	Leu	Pro 30	Pro	Pro	Pro	Glu	Val 35	Gly	Ser	Ile	Gln	Phe 40	Leu	Asn	Asp	Gln	1,72
	GCA	ATG	TAT	GAG	AAA	GGC	CGT	ATG	CTG	CGC	AAT	ACC	GAG	CGC	GGA	AAA	240
	Ala 45	Met	Tyr	Glu	Lys	Gly 50	Arg	Met	Leu	Arg	Asn 55	Thr	Glu	Arg	Gly	Lys 60	
10	22.5																
	CAG	GCA	CAG	GCA	GAT	GCT	GAC	CTG	GCC	GCA	GGG	GGT	GTG	GCA	ACC	GCA	288
					65	Ala				70					75		
	TTT	TCA	GGG	GCA	TTC	GGC	TAT	CCG	ATA	ACC	GAA	AAA	GAC	TCT	CCG	GAG	336
15				80		Gly			85					90			
	CTG	TAT	AAA	CTG	CTG	ACC	AAT	ATG	ATT	GAG	GAT	GCC	GGT	GAT	CTT	GCC	384
	Leu	Tyr	Lys 95	Leu	Leu	Thr	Asn	Met 100	Ile	Glu	Asp	Ala	Gly 105	Asp	Leu	Ala	
20	ACC	CGC	TCC	GCC	AAA	GAA	CAT	TAC	ATG	CGC	ATC	CGG	CCG	TTT	GCG	TTT	432
	Thr	Arg 110	Ser	Ala	Lys	Glu	His 115	Tyr	Met	Arg	Ile	Arg 120	Pro	Phe	Ala	Phe	
	TAC	GGC	ACA	GAA	ACC	TGT	AAT	ACC	AAA	GAT	CAG		AAA	СТС	TCC	ACC	480
	Tyr	Gly	Thr	Glu	Thr	Cys	Asn	Thr	Lys	Asp	Gln	Lys	Lys	Leu	Ser	Thr	300
25	125					130					135					140	
	AAC	GGA	TCT	TAC	CCG	TCA	GGT	CAT	ACG	TCT	ATC	GGC	TGG	GCA	ACC	GCA	528
	Asn	Gly	Ser	Tyr	Pro 145	Ser	Gly	His	Thr	Ser 150	Ile	Gly	Trp	Ala	Thr 155	Ala	
	CTG	GTG	CTG	GCG	GAA	GTG	AAC	CCG	GCA	AAT	CAG	GAT	GCG	ATT	CTG	GAA	576
30	Leu	Val	Leu	Ala 160	Glu	Val	Asn	Pro	Ala 165	Asn	Gln	Asp	Ala	Ile 170	Leu	Glu	
	ÇGG	GGT	TAT	CAG	CTC	GGA	CAG	AGC	CGG	GTG	ATT	TGC	GGC	TAT	JAC	TGG	624
	Arg	Gly	Tyr 175	Gln	Leu	Gly	Gln	Ser 180	Arg	Val	Ile	Cys	Gly 185	Tyr	His	Trp	, 52.
35	CAG	AGT	GAT	GTG	GAT	GCC	GCG	CGG	ATT	GTC	GGT	TCA	GCC	GCT	GTC	GCG	672
	Gln	Ser 190	Asp	Val	Asp	Ala	Ala 195	Arg	Ile	Val	Gly	Ser 200	Ala	Ala	Val	Ala	٠,٠
	ACA	TTA	CAT	TCC	GAT	CCG	GCA	TTT	CAG	GCG	CAG	TTA	GCG	AAA	GCC	AAA	720
40	Thr 205	Leu	His	Ser	Asp	Pro 210	Ala	Phe	Gln	Ala	Gln 215	Leu	Ala	Lys	Ala	Lys 220	
						AAA				TAA							750
	Gln	Glu	Phe	Ala	Gln 225	Lys	Ser	Gln	Lys 229			•					

INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 249 amino acids
 - (B) TYPE: amino acid

50

45

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(vi) ORIGINAL SOURCE:

5		(AT) OR		AL 50											
•	(A) ORGANISM: Morganella morganii(B) STRAIN: NCIMB 10466															
			(1													
		(xi) SE	QUEN	CE DI	ESCR	IPTI	ON:	SEQ	ID N	o:3:					
10	Met -20				Ile							Ser	Leu	Phe	Ser	Leu -5
	Ser	Ala	Leu	Ala	Ala 1	Ile	Pro	Ala	Gly 5	Asn	Asp	Ala	Thr	Thr 10	Lys	
15	Asp	Leu	Tyr 15	Tyr	Leu	Lys	Asn	Glu 20	Gln	Ala	Ile	Asp	Ser 25		Lys	Leu
	Leu	Pro 30	Pro	Pro	Pro	Glu	Val 35	Gly	Ser	Ile	Gln	Phe 40	Leu	Asn	Asp	Gln
20	Ala 45	Met	Tyr	Glu	Lys	Gly 50	Arg	Met	Leu	Arg	Asn 55	Thr	Glu	Arg	Gly	Lys 60
	Gln	Ala	Gln	Ala	Asp 65	Ala	Asp	Leu	Ala	Ala 70	Gly	Gly	Val	Ala	Thr 75	Ala
25	Phe	Ser	Gly	Ala 80	Phe	СĵА	Tyr	Pro	Ile 85	Thr	Glu	Lys	Asp	Ser 90		Glu
	Leu	Tyr	Lys 95	Leu	Leu	Thr	Asn	Met 100	Ile	Glu	Asp	Ala	Gly 105		Leu	Ala
	Thr	Arg 110	Ser	Ala	Lys	Glu	His 115	Tyr	Met	Arg	Ile	Arg 120	Pro	Phe	Ala	Phe
30	Tyr 125	Gly	Thr	Glu	Thr	Cys 130	Asn	Thr	Lys	Asp	Gln 135	Lys	Lys	Leu	Ser	Thr 140
	Asn	Gly	Ser	Tyr	Pro 145	Ser	Gly	His	Thr	Ser 150	Ile	Gly	Trp	Ala	Thr 155	
35				160	Glu				165					170		
	Arg	Gly	Tyr 175	Gln	Leu	Gly	Gln	Ser 180	Arg	Val	Ile	Cys	Gly 185	Tyr	His	Trp
40	Gln	Ser 190	Asp	Val	Asp	Ala	Ala 195	Arg	Ile	Val	Gly	Ser 200	Ala	Ala	Val	Ala
	205				Asp	210				Ala	Gln 215	Leu	Ala	Lys	Ala	Lys 220
45	Gln	Glu	Phe	Ala	Gln 225		Ser	Gln	Lys 229							
	T \	505)														
	INI				R SEC								•			
		(1)			CE CH											
50					ENGTH				acio	ds						
					PE:											
					OPOLO											
					LE TY			tein								
		(vi)	OR	IGINA	AL SC	DURCE	Ξ:	•								
55																

	(A) ORGANISM: Morganella morganii (B) STRAIN: NCIMB 10466															
	(B) STRAIN: NCIMB 10466 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:															
5	71-	(XI) SE	OUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:4:				·	
	1	Ile			5					10					15	
		Lys		20					25					30		
10		Glu	35					40					45			
	Lys	Gly 50	Arg	Met	Leu	Arg	Asn 55	Thr	Glu	Arg	Gly	Lys 60	Gln	Ala	Gln	Ala
15	Asp 65	Ala	Asp	Leu	Ala	Ala 70	Gly	Gly	Val	Ala	Thr 75		Phe	Ser	Gly	Ala 80
	Phe	Gly	Tyr	Pro	Ile 85	Thr	Glu	Lys	Asp	Ser 90		Glu	Leu	Tyr	Lys 95	Leu
20	Leu	Thr	Asn	Met 100	Ile	Glu	Asp	Ala	Gly 105		Leu	Ala	Thr	Arg 110	Ser	Ala
		Glu	115					120					125			
25		Cys 130					135					140				_
	145	Ser				150					155					160
30		Val			165					170					175	
		Gly		180					185					190		
		Ala	195					200					205			
35		Pro 210					Gln 215	Leu	Ala	Lys	Ala	Lys 220	Gln	Glụ	Phe	Ala
	Glr 225	ı Lys 5	s Sei	Glr	Lys 229											
40																
	INE	FORMA (i)	SEÇ	V FOR QUENC A) LE	E CF	IARAC	TER	STIC		•						
45			(E	3) TY	PE:	amir	o ac	id	clas	5						
			MOI	ECUI	E TY	PE:	pept	ide								
		(v) (vi)	ORI	COTEI	L SC	URCE	:									
50			(E	A) OF	'RAIN	1: JC	M 16	50								
	.	(xi)	SEC	UENC	E DE	SCRI	PTIC	N: S	EQ I	D NC	:5:					
	Leu 1	Ala	ren	Val	Ala 5	Thr	Gly	Asn	Asp	Thr 10	Thr	Thr	Lys	Pro	Asp 15	Leu
55																

	INE	ORMA	TION	FOF	SEC	DI	NO:	5 :									
				UENC					cs:								
				L) LE						s					•		
5				3) TY													
			(0	;) SI	RANI	DEDNE	ESS:	doub	ole								
			(I) TC	POL	GY:	line	ear									
		(ii)	MOI	ECUI	E TY	PE:	gend	omic	DNA								
	((iii)	HYP	POTHE	TICA	T: 1	10										
10		(iv)	ANT	I-SE	NSE:	NO											
		(vi)	ORI	GINA	L SC	DURCE	Ξ:										
			(F	A) OF	RGAN	SM:	Esch	nerio	chia	blat	tae						
			(E	3) SI	RAI	1: J	M 16	650									
		(ix)	FEA	ATURE	Ξ:												
15			(J	A) NA	ME/F	KEY:	CDS										
			(E	3) LC	CAT	ON:	1	747									
		(ix)		ATURE													
				A) NA					ide								
				3) LO		ION:	1	54									
20		(ix)		ATURE			_										
				A) NA					Lde								
		977		3) LO					- D 17								
				CE DE							666	667	mmc	mm.c	mam		4.0
25				CGT													48
	-18	тÃ2	гуѕ	Arg -15	vai	Leu	міа	vai	-10	Pne	Ala	Ата	Leu	-5	ser	Ser	
		GCC	CTG	GCG	СТС	GTC	сст	ACC	-	מממ	GAC	лст	A C C	_	אאא	CCC	96
				Ala													96
	GIII	лта	1	AIG	Бец	Val	5	1111	GLY	MOII	voh	10	1111	1111	пуз	FLO	
30	GAT	СТС		TAC	СТС	AAG		AGT	GAA	GCC	α T T		A'GC	СТС	ccic	CTG	144
				Tyr													***
	15			- 4 -		20					25					30	
	TTG	CCG	CCA	CCA	CCG	GCG	GTG	GGC	TCC	ATT		TTT	CTC	AAC	GAT		192
				Pro													
35					35			_		40					45		
	GCC	ATG	TAT	GAA	CAG	GGG	CGC	CTG	CTG	CGC	AAC	ACC	GAA	CGC	GGT	AAG	240
	Ala	Met	Tyr	Glu	Gln	Gly	Arg	Leu	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Lys	
				50					55					60			
40	CTG	GCG	GCG	GAA	GAT	GCA	AAC	CTG	AGC	AGT	GGC	GGG	GTG	GCG	AAT	GCT	288
40	Leu	Ala		Glu	Asp	Ala	Asn	Leu	Ser	Ser	Gly	Gly	Val	Ala	Asn	Ala	
		,	65					7 (. 79				
				GCG													336
	Phe		Gly	Ala	Phe	Gly		Pro	Ile	Thr	Glu	_	Asp	Ala	Pro	Ala	
45		80					85					90					
				TTA													384
		HIS	ьys	Leu	Leu		Asn	met	ııe	Glu		Ala	Gly	Asp	Leu		
	95	~~~	3.66	GCG		100	a	m » —		~~~	105	~~-				110	
	ALC:	t tal.	ALT.	(-([-	A A A	LAAT	L.AL.	LAT	A 116	((-(A T''	1 (51)	CCC	יוייני.	1:176	THE	432

	Thr	Arg	Ser	Ala	Lys 115	Asp	His	Tyr	Met	Arg 120		Arg	Pro	Phe	Ala 125	Phe	
	TAT	GGG	GTC	TCT	ACC	TGT	AAT	ACC	ACC			GAC	AAA	CTG	TCC	מממ.	480
5	Tyr	Gly	Val	Ser	Thr	Cvs	Asn	Thr	Thr	Glu	Gln	Asp	Lvs	Len	Ser	Luc	400
	•	-		130		- 4 -			135			op	2,0	140	JCI	Lys	
	AAT	GGC	TCT	TAT	CCG	TCC	GGG	CAT		тст	ATC	GGC	TGG	CCT	ACT	GCG	500
	Asn	Gly	Ser	Tvr	Pro	Ser	Glv	His	Thr	Ser	TIA	Glv	Trn	מכו	The	71.5	528
		,	145	-1-		001	O-7	150	* * * * *	561	116	Gry	155	Ala	1111	AIG	
10								100					133				
	CTG	GTG	CTG	GCA	GAG	ATC	AAC	CCT	CAG	CGC	CAG	AAC	GAG	ATC	СТС	מממ	576
	Leu	Val	Leu	Ala	Glu	Ile	Asn	Pro	Gln	Ara	Gln	Asn	Glu	Tla	Leu	Tue	3/0
		160					165			9	02	170	Ciu	116	Deu	пуз	
	CGC	GGT	TAT	GAG	CTG	GGC		AGC	CGG	GTG	ΑTT		GĠC	TAC	CAC	TGG	624
15	Arg	Gly	Tvr	Glu	Leu	Glv	Gln	Ser	Ara	Val	Tle	Cvs	GUU	Tur	Uic	TGG	624
	175	•	-			180			9		185	CyS	GTA	IAT	nis	11p	
	CAG	AGT	GAT	GTG	GAT		GCG	CGG	GTA	GTG		ጥርጥ	GCC	CTT	CTC	190	c 7 0
	Gln	Ser	Asp	Val	Asp	Ala	Ala	Ara	Val	Val	GUA	Ser	מככ	Gil	V-1	37.	672
			F -		195			9	• • •	200	Gry	261	VIG	Vai		мта	
20	ACC	CTG	CAT	ACC		CCG	GCG	TTC	CAC		CAC	mmc.	CAC		205		
	Thr	Leu	His	Thr	Asn	Pro	2 l a	Pho	CAG	CAG	CAG	Tan	CAG	AAA	31-	AAG	720
		200	1.10	210	ASII	110	VIG	FIIE	215	GIII	GIII	ьeu	GIN		Ala	rys	
	GCC	GAA	ጥጥር		CAG	СЪТ	CAG	D D C		ጥአአ				220			
	Ala	Glu	Phe	Ala	Gln	Wie	Gln	Luc	Tuc	IAA							750
25		0.10	225	71.2.0	G1 11	1113	GIII	230	гур								
			223					230									
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		,		A) OF				eric	hia	blat	tae						
35				3) S7						~~~	·cac						
		(xi)	SEC						EO I	ם אכ	.7.						
	Met	Lys	Lys	Arg	Val	Leu	Ala	Val	Cvs	Phe	Ala	Ala	T.eu	Phe	Sar	Sar	
	-18	-	•	-15					-10			1110	Deu	-5	Ser	Ser	
	Gln	Ala	Leu	Ala	Leu	Val	Ala	Thr		Asn	Asn	Thr	ጥኩ ∽	Th~	Tuc	Dwa	
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	Asp	Leu	Tvr	Tvr	Leu	Lvs	Asn	Ser	Glu	Δla	Tla		505	T 011	70.7	Y	
	15		- 4 -			20		001	GIU	ALG	25	N311		Lea	AId		
	Leu	Pro	Pro	Pro	Pro		Val	Glv	Sar	Tla		Pho	Lou	7	7	30	
					35		•	O.J.	DCI	40	VIG	rne	Leu	ASII		GIU	
45	Ala	Met	Tvr	Glu		Glv	Ara	I.eu	T.e.n		Acn	Thr	C1	7. va ~	45	*	
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	Leu	Ala	Ala		Asp	Ala	Asn	Len		502	Gly	G) v	3/ - 1	60 71 a	7	7.1	
			65		p			70	JGL	261	GIY	Gry			ASN	мта	
								, 0					75				

	Phe	Ser 80	Gly	Ala	Phe	Gly	Ser 85	Pro	Ile	Thr	Glu	Lys 90	Asp	Ala	Pro	Ala
5	Leu 95	His	Lys	Leu	Leu	Thr 100	Asn	Met	Ile	Glu	Asp 105	Ala	Gly	Asp	Leu	Ala 110
	Thr	Arg	Ser	Ala	Lys 115	Asp	His	Tyr	Met	Arg 120	Ile	Arg	Pro	Phe	Ala 125	Phe
10	Tyr	Gly	Val	Ser 130	Thr	Cys	Asn	Thr	Thr 135	Glu	Gln	Asp	Lys	Leu 140	Ser	Lys
	Asn	Gly	Ser 145	_	Pro	Ser	Gly	His 150	Thr	Ser	Ile	Gly	Trp 155	Ala	Thr	Ala
15	Leu	Val 160	Leu	Ala	Glu	Ile	Asn 165	Pro	Gln	Arg	Gln	Asn 170	Glu	Ile	Leu	ГÀ2
	Arg 175	Gly	Tyr	Glu	Leu	Gly 180	Gln	Ser	Arg	Val	Ile 185	Cys	Gly	Tyr	His	Trp 190
20		Ser	_		195					200	_				205	
		Leu		210					215	Gln	Gln	Leu	Gln	Lys 220	Ala	Lys
25	Ala	Glu	Phe 225	Ala	Gln	His	Gln	Lys 230	Lys							
	IN	FORM	ATIO	v FOI	R SE	Q ID	NO:	8:								
		(i)) SE(_	CE CI ENGTI					ds						
30					YPE: OPOLO				1							
) MO	LECU	LE T	YPE:	pro						,			
35		(• ±	(2	(A)	RGAN:	ISM:	Esc		chia	bla	ttae		•			
		(xi) SE						SEQ :	ID N	D:8:					
	Leu 1	Ala	Leu	Val	Ala 5	Thr	Gly	Asn	Asp	Thr 10	Thr	Thr	Lys	Pro	Asp 15	Leu
40	Tyr	Tyr	Leu	Lys 20	Asn	Ser	Glu	Ala	Ile 25	Asn	Ser	Leu	Ala	Leu 30	Leu	Pro
	Pro	Pro	Pro 35	Ala	Val	Gly	Ser	Ile 40	Ala	Phe	Leu	Asn	Asp 45	Gln	Ala	Met
45	Tyr	Glu 50	Gln	Gly	Arg	Leu	Leu 55	Arg	Asn	Thr	Glu	Arg 60	Gly	Lys	Leu	Ala
	Ala 65	Glu	Asp	Ala	Asn	Leu 70	Ser	Ser	Gly	Gly	Val 75	Ala	Àsn	Ala	Phe	Ser 80
50	Gly	Ala	Phe	Gly	Ser 85	Pro	Ile	Thr	Glu	Lys 90	Asp	Ala	Pro	Ala	Leu 95	His
	Lys	Leu	Leu	Thr 100	Asn	Met	Ile	Glu	Asp 105	Ala	Gly	Asp	Leu	Ala 110	Thr	Arg
55	Ser	Ala	Lys	Asp	His	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	Tyr	Gly

			115					120					125					
		Ser 130					135					140		_			٠.	
5	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	Leu	Val		
	145					150					155					160		
	Leu	Ala	Glu	Ile	Asn 165	Pro	Gln	Arg	Gln	Asn 170	Glu	Ile	Leu	Lys	Arg 175	Gly		
	Tyr	Glu	Leu	Gly	Gln	Ser	Arg	Val	Ile		Glv	Tvr	His	Trp		Ser		
10				180			-		185	-	•			190				
	Asp	Val	Asp 195	Ala	Ala	Arg	Val	Val 200	Gly	Ser	Ala	Val	Val 205	Ala	Thr	Leu		
	His	Thr 210	Asn	Pro	Ala	Phe	Gln 215	Gln	Gln	Leu	Gln	Lys 220	Ala	Lys	Ala	Glu		
15	Phe	Ala	Gln	His	Gln	Lvs						220						
	225	••••	· · · ·		01	230	טעט											
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25		(iii)								y i i C	16616	DIV	1					
			AN				••							•				
			SEC				PTI	ON: S	SEO :	ID NO	0:9:							
	CCT	CGAG																20
30	IN	FORM	OITA	1 FOI	R SE	Q ID	No:	10:										
			SEC						CS:									
				A) LI				se p		5								
			(E	3) T	PE:	nucl	leic	acio	i									
			((C) 5:	rani	DEDNE	ESS:	sing	jle									
35						OGY:												
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		(iii)																
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40	3.00		SEC				PTIC	on: s	EQ :	ID NO	0:10:							
40	ATT	CGCCF	ACA :	rcgcc	CACTO	SC T												21
	IN	FORMA	OITA	1 FOE	R SE	QI C	NO: 1	11:										
		(i)	SEC															
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						GY:											٠	
		(ii)	MOI	LECUI	LE T	PE:	othe	er DN	JA	ynth	netic	: DNA	1					
50																		
														*				

	(iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO	
5	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11: TAGCCCAGCC GGTAGAGGTA TG	22
	INFORMATION FOR SEQ ID NO:12: (i) SEQUENCE CHARACTERISTICS:	
10	(A) LENGTH: 23 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear	
15	<pre>(ii) MOLECULE TYPE: other DNAsynthetic DNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12: TGCATCTGCC TGCGCCTGCT TAC</pre>	23
20	INFORMATION FOR SEQ ID NO:13: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	
25	(D) TOPOLOGY: linear (ii) MOLECULE TYPE: other DNAsynthetic DNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
30	AACGCGCCGT AGAAAGCATT	20
35	INFORMATION FOR SEQ ID NO:14: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
40	(ii) MOLECULE TYPE: other DNAsynthetic DNA (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14: GTCCTGGTCT TTGGTATTAC A	21
45	INFORMATION FOR SEQ ID NO:15: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	
50	(D) TOPOLOGY: linear	

(iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15: CACATCGCCA GCGGCCAGGT CTGCAT INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	26
CACATCGCCA GCGGCCAGGT CTGCAT INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	26
INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	26
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	
(A) LENGTH: 21 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	
(B) TYPE: nucleic acid(C) STRANDEDNESS: single	
(C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: other DNAsynthetic DNA	
15 (iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
GCATATAGTG TTCTTTCGCG C	21
20 INFORMATION FOR SEQ ID NO:17:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 22 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	
25 (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: other DNAsynthetic DNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
ATTACAGGTT TCGACCCCAT AA	22
INFORMATION FOR SEQ ID NO:18:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 25 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: other DNAsynthetic DNA	
(iii) HYPOTHETICAL: NO	•
(1V) ANTI-SENSE: NO	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:	
TGATGCATGT CCGGGCTGTC TTTTT	.25
INFORMATION FOR SEQ ID NO:19:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 25 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	

				LECU				er D	NA	synt	heti	c DN	Ά				
				POTH													
				TI-S													
5				QUEN					SEQ	ID N	0:19	:					
	CTG	GATC	CTG	TGGC	TATC	AT C	ACCT										25
	TN	FORM	חדת	N FO	D CE	Λ T D	NO.	20.									
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10		, -		A) L													
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				c) s													
				D) T					3 -0								
		(ii		LECU					NA	svnt	heti	c DN	A				
15				POTH						•			- •				
		(iv) AN	TI-S	ENSE	: NO											
		(xi) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:16	:					
	CTG	GATC	CGA	CGCG.	ATTT	TA C	CATA										25
20	IN			N FO													
		(i		QUEN													
				A) L						s ·							
				B) T													
25				C) S					ple								
		/::		D) T					D.177								
				LECU: POTH				omic	DNA								
				TI-S			NO										
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30		(A) O				wide	ncia	etn	arti	i					
				B) S'						scu	arti	т					
		(ix		ATURI					-		,						
	•	·		A) N		KEY:	CDS										
				B) L				744									
35	(xi) SE	QUEN	CE DI	ESCR.	IPTI	ON:	SEQ	ID N	0:21	:						
	ATG	AAA	AAA	CTA	TTA	GCA	GTA	TTC	TGC	GCA	GGG	GCT	TTT	GTT	TCA	ACC	48
	Met	Lys	Lys	Leu	Leu	Ala	Val	Phe	Cys	Ala	Gly	Ala	Phe	Val	Ser	Thr	
	1				5					10					15		
40	AGT	GTA	TTT	GCG	GCG	ATC	CCT	CCC	GGC	AAT	GAT	GTG	ACA	ACT	AAA	CCC	96
	Ser	Val	Phe	Ala	Ala	Ile	Pro	Pro		Asn	Asp	Val	Thr	Thr	Lys	Pro	
	C D III	a m m		20					25					30			
	GAT	CTT	TAT	TAT	TTA	AAA	AAC	TCA	CAG	GCT	ATT	GAT	AGT	TTA	GCG	TTA	144
	ASp	rea		Tyr	ьeu	гÀг	Asn		Gln	Ala	Ile	Asp		Leu	Ala	Leu	
45	ттс	ccc	35	CCA	CCM	C B B	CIDC	40	B. C. M.	3.00			45				
	Len	Pro	Pro	CCA	CCI	Clu	GIG	C1	AGT	ATC	TTA	TTT	TTA	AAC	GAC	CAA	192
	~cu	50		Pro	FIO	GIU	55	GTÅ	ser	тте	neu		ьeu	Asn	Asp	GIn	
	GCG		TAT	GAA	ממג	GGC		αጥጥ	ው ምር	CGD	יים מב	60 ACT	CNC	CCT	CCT	~ ~ ~	0.4.0
				J. 21	• • •	<u> </u>		***	110	CGM	VVI.	VCI	GAG	CGI	GGA	GAA	240
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	Δla	Met	Tur	Glu	Luc	Glv	Ara	T.eu	Len	Ara	Asn	Thr	Glu	Ara	Glv	Glu		
	65	1166	- y -	Olu	БУЗ	70	ALG	Бец	Deu	ALG	75	1111	Giu	AL 9	Gry	80		
_	CAA	GCC	GCT	AAG	GAT	GCT	GAT	CTG	GCT	GCG	GGC	GGT	GTT	GCG	AAC	GCA	288	3
5	Gln	Ala	Ala	Lys	_	Ala	Asp	Leu	Ala		Gly	Gly	Val	Ala		Ala		
	~~~	m.c.m	~ n n	CCM	85	CC#	m	CCC	3 mm	90	C	B B C	C 3 M	666	95	GD D	2.24	_
		TCT Ser															336	2
	File	Ser	Giu	100	riie	GIY	ıyı	FIU	105	1111	GIU	БÃЗ	ASP	110	PIO	Gru		
10	ATT	CAT	AAA		CTG	ACG	AAT	ATG	-	GAA	GAT	GCG	GGG		TTA	GCA	384	4
		His																
			115					120					125					
		CCC															432	2
15	Thr	Arg 130	Ser	Ala	ьys	GIU	ьуs 135	Tyr	мет	Arg	ile	Arg 140	Pro	Phe	Ala	Phe		_
	TAC	GGT	GTT	GCT	ACC	ፐርፐ		ACG	AAA	GAT	CAG	-	מממ	ттъ	ጥሮጥ	AAG	480	n.
		Gly															100	
	145	-				150			-	•	155	•	-			160		
20		GGC															528	3
	Asn	Gly	Ser	Tyr		Ser	Gly	His	Thr		Ile	Gly	$\mathtt{Trp}$			Ala		
	ama		mm.a	mar.	165	7 mm			a	170	~~~				. 175			_
		GTA															576	ó
25	Leu	Val	ьeu	180	GIU	TIE	ASII	PLO	185	ASII	GIII	Asp	гус	190	ьеu	rys		
25	CGT	GGT	TAT		CTT	GGC	CAA	AGC		GTC	ATC	TGT	GGT		CAT	TGG	624	4
		Gly																
			195					200					205					
		AGT															672	2
30	Gin	Ser 210		vaı	Asp	Ата	A1a 215	Arg	11e	vaı	Ala	220	GIŸ	Ala	Val	Ala		
	ACT	TTA		TCC	AAC	ССТ		TTC	CAA	AAA	CAG		CAA	ΔΑΔ	GCC	<b>44</b>	720	<u></u>
		Leu															, 2 \	,
	225					230				-	235					240		
<i>35</i>		GAA							TAG								74	7
•	Asp	Glu	Phe	Ala	_	Leu	Lys	Lys										
					245													
	IN	FORM	ATIO	N FO	R SE	Q ID	NO:	22:										
40			) SE						CS:									

- - (A) LENGTH: 248 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Providencia stuartii
  - (B) STRAIN: ATCC 29851
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Met Lys Lys Leu Leu Ala Val Phe Cys Ala Gly Ala Phe Val Ser Thr

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	. 1				5					10					15	
			Phe	20					25					30.	Lys	
5			Tyr 35					40					45			
	Leu	Pro 50	Pro	Pro	Pro	Glu	Val 55	Gly	Ser	Ile	Leu	Phe 60	Leu	Asn	Asp	Gln
10	65		Tyr			70			•		75				_	80
	Gln	Ala	Ala	Lys	Asp 85	Ala	Asp	Leu	Ala	Ala 90	Gly	Gly	Val	Ala	Asn 95	Ala
15	Phe	Ser	Glu	Ala 100	Phe	Gly	Tyr	Pro	Ile 105	Thr	Glu	Lys	Asp	Ala 110	Pro	Glu
	Ile	His	Lys 115	Leu	Leu	Thr	Asn	Met 120	Ile	Glu	Asp	Ala	Gly 125	Asp	Leu	Ala
20	Thr	Arg 130	Ser	Ala	Lys	Glu	Lys 135	Tyr	Met	Arg	Ile	Arg 140	Pro	Phe	Ala	Phe
	145		Val			150					155					160
25			Ser		165					170					175	Ala
25			Leu	180					185					190		
	Arg	Gly	Tyr 195	Glu	Leu	Gly	Gln	Ser 200	Arg	Val	Ile	Cys	Gly 205	Tyr	His	Trp
30		210	Asp				215					220				
	Thr 225	Leu	His	Ser	Asn	Pro 230	Glu	Phe	Gln	Lys	Gln 235	Leu	Gln	Lys	Ala	Lys 240
35	Asp	Glu	Phe	Ala	Lys 245	Leu	Lys	Lys								
	INE	FORMA (i)	ATION SEC			) ID LARAC			·c •							
40		, _ /			NGTH			ase		S						

- (A) LENGTH: 744 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Enterobacter aerogenes
  - (B) STRAIN: IFO 12010
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 1..744

	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:  ATG AAA AAG CGC GTT CTC GCC CTC TGC CTC GCC AGC CTG TTT TCC GTT  48  Met Lys Lys Arg Val Leu Ala Leu Cys Leu Ala Ser Leu Phe Ser Val																	
	ATG	AAA	AAG	CGC	GTT	CTC	GCC	CTC	TGC	CTC	GCC	AGC	CTG	TTT	TCC	GTT		48
	Met	Lys	Lys	Arg	Val	Leu	Ala	Leu	Cys	Leu	Ala	Ser	Leu	Phe	Ser	Val		
5	1				5					10					15			
	AAC	GCT	TTC	GCG	CTG	GTC	CCT	GCC	GGC	AAT	GAT	GCA	ACC	ACC	AAA	CCG		96
	Asn	Ala	Phe	Ala	Leu	Val	Pro	Ala	Gly	Asn	Asp	Ala	Thr	Thr	Lys	Pro		
				20					25					30	-			
	GAT	CTC	TAT	TAT	CTG	AAA	AAT	GCA	CAG	GCC	ATC	GAT	AGT	CTG	GCG	CTG	1	44
10	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ala	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu		
			35					40					45					
	TTG	CCG	CCG	CCG	CCG	GAA	GTT	GGC	AGC	ATC	GCA	TTT	TTA	AAC	GAT	CAG		192
	Leu	Pro	Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Ala	Phe	Leu	Asn	Asp	Gln		
		50					55					60						
15	GCG	ATG	TAT	GAG	AAA	GGA	CGG	CTG	TTG	CGC	AAT	ACC	GAA	CGT	GGC	AAG	2	240
	Ala	Met	Tyr	Glu	Lys	Gly	Arg	Leu	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Lys		
	65					70					75					80		
		GCG															2	288
	Leu	Ala	Ala	Glu	Asp	Ala	Asn	Leu	Ser	Ala	Gly	Gly	Val	Ala	Asn	Ala		
20					85			•		90					95			
		TCC															;	336
	Phe	Ser	Ser		Phe	Gly	Ser	Pro	Ile	Thr	Glu	Lys	Asp	Ala	Pro	Gln		
				100					105					110				
		CAT															:	384
25	Leu	His	_	Leu	Leu	Thr	Asn		Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala		
			115					120					125					
		CGC															4	132
	Thr	Arg	Ser	Ala	Lys	Glu		Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe		
		130					135					140						
30		GGC															4	480
		Gly	Val	Ser	Thr		Asn	Thr	Thr	Glu		Asp	Lys	Leu	Ser	-		
	145					150					155					160		
	77.	GGA	T C T	መክር	CCT	mcc		~ n ~	7.00	m c m	70.00	CCT	шсс	CC3	7.00	cac		- 2 0
35		Gly																528
	ASII	GIY	Jer	ıyı	165	361	GIÅ	1115	1111	170	116	GIY	тър	MId	175	MIG		
	СТС	GTA	CTG	GCG		<b>ልጥ</b> ር	ד מ מ	CCG	CAG		$\alpha$	D D C	CDD	TA TO TO		מתה		576
		Val															•	370
	Deu	Val	Deu	180	Olu	116	risii	110	185	ALG	GIII	NSII.	Giu	190	пеа	гуз		
40	CGC	GGC	тат		TTG	GGC	GAA	AGC		СТТ	ATC	TGC	GGC		СУТ	TGG	4	624
		Gly															`	029
	9	017	195	014	204	Cry	024	200	9	VUI		Cys	205	1 7 1	1113	115		
	CAG	AGC		GTC	GAT	GCG	GCG		ATA	GTC	GGC	TCG		GTG	GTG	GCG	6	672
		Ser															`	
45		210					215				1	220						
	ACC	CTG	CAT	ACC	AAC	CCG		TTC	CAA	CAG	CAG		CAG	AAA	GCA	AAG		720
,		Leu																
	225					230			- · <del>-</del>		235			-3-		240		
						-					-							

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GAT GAA TTC GCC AAA ACG CAG AAG TAA

	Asp	Glu	Phe	Ala	Lys 245	Thr	Gln	Lys								
5	~~~	T00.41														
	TN.	FORM														
		(1)		QUEN( A) Li						4.5						
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10				D) T(												
,,		(ii)	MO	LECUI	LE T	YPE:	prot	cein								
		(vi)		IGIN												
				A) OI					acte	r aei	rogei	nes				
		14:1		3) Si						FD 17						•
15	Met	Lys	Tus	OUENC Ara	ve Di Val	LOU	D) =	Len	CVC.	ID NO	7:24		T 0.11	Dho	C	17- 1
	1	-,-	_,_	9	5	200	1114	Dea	Cys	10	ALA	Ser	теп	rne	15	vaı
	Asn	Ala	Phe	Ala 20	Leu	Val	Pro	Ala	Gly 25	Asn	Asp	Ala	Thr			Pro
20	Asp	Leu	Tvr		Leu	Lvs	Asn	Ala		Ala	T16	Aen	Sar	30	ת ו ת	Lou
			35					40					45			
		Pro 50					55					60				
25	Ala 65	Met	Tyr	Glu	Lys		Arg	Leu	Leu	Arg		Thr	Glu	Arg	Gly	Lys
20		Ala	Ala	Glu	Asn	70 11a	Δen	Tan	502	712	75	C1	17- 1	71.	7	80
					85					90					95	
	Phe.	Ser	Ser	Ala 100	Phe	Gly	Ser	Pro	Ile 105	Thr	Glu	Lys	Asp	Ala 110	Pro	Gln
30	Leu	His	Lys		Leu	Thr	Asn	Met		Glu	Asp	Ala	Glv		Leu	Ala
			115					120					125			
	Thr	Arg	Ser	Ala	Lys	Glu		Tyr	Met-	Arg	Ile		Pro	Phe	Ala	Phe
	Tur	130	Val	502	<b>ም</b> ኮ ~	Cuc	135	Th ω	mb -	C1	<b>~1</b> -	140			_	_
35	145	Gly	vai	261	1111	150	NSII	1111	1111	GIU	155	Asp	гÀг	Leu	Ser	Lys 160
		Gly	Ser	Tyr	Pro		Gly	His	Thr	Ser		Glv	Trp	Ala	Thr	Ala
					165					170					175	
	Leu	Val	Leu	Ala	Glu	Ile	Asn	Pro	Gln	Arg	Gln	Asn	Glu	Ile	Leu	Lys
40	7	C1	m	180		~,	_,	_	185					190		
	Arg	Gly	Tyr 195	GLu	Leu	Gly	Glu	Ser 200	Arg	Val	Ile	Cys	Gly 205	Tyr	His	Trp
	Gln	Ser	Asp	Val	Asp	Ala		Arg	Ile	Val	Gly	Ser	Ala	Val	Val	Ala
	ጥሎ	210	*** =	m <b>L</b>	<b>n</b> -	ъ.	215	_,				220				
45	225	Leu	HIS	Inr	Asn	Pro 230	Ala	Phe	Gln	Gln		Leu	Gln	Lys	Ala	_
		Glu	Phe	Ala	Lvs		Gln	Lvc			235					240.
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		(i		QUEN													
5				A) L			747			rs						•	
J				B) T								,					
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15		(17				vev.	CDC										
				A) N. B) L				717									
	(vi	) SE							TD M	0.25	_						
	ATG	AAA	DAG	CGT.	CTA		GCC	ும்ம நெழி	דט מי	CTTT		7.00	cm.c	<b></b>			
00	Met	Lys	Lvs	Ara	Val	T.O.II	Ala	Ton	Cvc	CII	אוה	AGC	UTC	TTT	TCA	GTT	48
20	1	_,	2,5	2119	5	Бец	VITO	neu	Cys	10	Ald	ser	reu	Pne		Val	
	_	GCC	ттт	GCG	-	GTT	כככ	GCC	GGC		СЛТ	ccc	700	N.C.C	15	ccc	0.0
	Ser	Ala	Phe	Ala	Leu	Val	Pro	Ala	Glv	Asn	Asp	Ala	Thr	Thr	Lve	Pro	96
				20	-				25				****	30	цуз	110	
25	GAT	CTC	TAC	TAT	CTG	AAA	AAT	GCC	CAG	GCC	ATT	GAC	AGC	CTG	GCG	CTG	144
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ala	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu	131
			35					40					45				
							•										
	ጥጥረ	CCA	ccc	ccc	ccc	~ n n	c m c	666									
30	T.A11	CCA	Pro	Pro	Dro	Clu	GIG	GGC	AGC	ATT	GCG	TTT	TTA	AAC	GAT	CAG	192
	Dea	Pro 50	110	110	FIO	Giu	55	GIA	ser	тте	ATG		Leu	Asn	Asp	GIn	
	GCG	ATG	тат	GAG	AAA	GGC		СТС	CTG	ccc	CCC	60 7.CC		CCC	CCC	770	
	Ala	Met	Tvr	Glu	Lvs	Glv	Ara	Len	Leu	Ara	Ala	Thr	NI a	75~	C1	AAG	240
35	65		- 4 -		-,, -	70	9		204	1119	75	1111	ATG	ALG	Gry	80 TAS	
	TTG	GCG	GCA	GAA	GAT	GCC	AAC	CTG	AGC	GCG		GĞC	GTG	GCC	AAC	GCC	288
	Leu	Ala	Ala	Glu	Asp	Ala	Asn	Leu	Ser	Ala	Glv	Glv	Val	Ala	Asn	Ala	200
					85					90	•	-			95		
	TTC	TCC	GCA	GCA	TTC	GGC	TCC	CCG	ATC	AGC	GAA	AAA	GAC	GCC	CCG	GCG	336
40	Phe	Ser	Ala	Ala	Phe	Gly	Ser	Pro	Ile	Ser	Glu	Lys	Asp	Ala	Pro	Ala	
				100					105					110			
	CTG	CAC	AAA	CTG	CTC	ACC	AAC	ATG	ATT	GAA	GAC	GCG	GGC	GAT	CTG	ĞCG	384
	Leu	His	Lys	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala	
			115					120					125				
45	ACC	CGA	GGC	GCG	AAA	GAG	AAG	TAT	ATG	CGT	ATT	CGT	CCG	TTT	GCC	TTC	432
	Thr	Arg	GTA	Ala	Lys	Glu	Lys	Tyr	Met	Arg	Ile		Pro	Phe	Ala	Phe	
	<b>ሞአ</b> ሮ	130	CEC	TCC	n	mcc.	135	200				140					
	THU	Glv	UTG Wal	100	ACC	TGC	AAT	ACC	ACC	GAA	CAG	GAT	AAG	CTG	TCG	AAA	480
	IVI	- T - V	v a ı	-3 H C	inr	1 7/5	A S 11	I D P	1.0 ~	f = 1 13	/ T   SA	0.00	1 150	T	C	<b>T</b> .	

	145					150					155					160	
	AAC	GGC	TCC	TAC	CCT	TCC	GGA	CAC	ACC	TCT	ATC	GGC	TGG	GCG	ACC	GCC	528
5						Ser											
	CTG	GTG	CTG	GCC		ATC.	AAC	CCG	CAG		CAG	דעם	GAG	ΔTT		אאכ	576
						Ile											370
				180			:		185		<b>Q1</b>	11011	Oru	190	Dea	БуЗ	
	CGC	GGC	TAT		CTC	GGT	GAA	AGT			ATC	TGC	GGT		CAC	ፐርር	624
10						Gly											021
	•	- 3	195			3		200	9			-,-	205	- 1 -			
	CAG	AGC	GAT	GTT	GAC	GCC	GCG	CGG	ATT	GTC	GGC	TCG	GCG	GTG	GTT	GCA	672
						Ala											
		210	-		_		215	•			-	220					
15	ACC	CTG	CAT	ACC	AAT	CCG	GCC	TTC	CAG	CAG	CAG	CTG	CAA	AAA	GCC	AAA	720
	Thr	Leu	His	Thr	Asn	Pro	Ala	Phe	Gln	Gln	Gln	Leu	Gln	Lys	Ala	Lys	
	225					230					235			-		240	
	GAC	GAG	TTT	GCG	AAA	CAG	CAG	AAA	TAG								747
	Asp	Glu	Phe	Ala	Lys	Gln	Gln	Lys									
20					245												
25			SE( () ()	QUENC A) LI B) T	CE CI ENGTI PE:	Q ID HARAG H: 26 amin	CTER 48 ar no ac	ISTIC mino cid		ds							
						OGY:											
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		(VI)				OURCI	_	, ·		_		_					
30						ISM:			IIa 1	plan	tico.	La					
		/ ÷ \				N: II			000	TD 31			•				
	Mat					ESCR:								-	~		
	met 1	гÀ2	ьys	Arg	vai 5	Leu	Ата	Leu	СУБ		АТА	Ser	Leu	Pne		Val	
		70.1 -	Dho	7.1.	_	77-1	D~o	ח 1 ח	C1	10	.7	71-	m\	m \	15	D	
35	Ser	MIG	FIIE	20	пеп	Val	PIO	Ald	25	ASII	ASP	Ala	Inr	30	гÀг	Pro	
	Asn	Len	Tur		T.em	Lys	A sp	Δ1 a		בות	Tlo	7 cn	S~*		71-	T ou	
	ıwp	Dea	35	1 Y L	Бец	Dys	ASII	40	GIII	ATA	116	Asp	45	Leu	Ala	reu	
	Leu	Pro		Pro	Pro	Glu	Wa l		Sar	Tlo	Δ1 a	Pho		7 cn	Λαν	Cln	
		50		110		0+4	55	013	Ser	116	AIG	60	Deu	ASII	ASP	GIII	
40	Ala		Tvr	Glu	I.vs	Gly		I.e.11	T.e.11	Ara	בומ		Δla	λτα	Gly	Luc	
	65	••••	-1-	010	-,-	70	•9	200	LCu	1119	75	1111	AT a	ALG	Gry	80	
		Ala	Ala	Glu	Asp	Ala	Asn	T.em	Sar	Δla		Gly	tia 1	7A 1 -s	Asn		
					85				5,1	90	<b>01</b>	Cry	٧۵١	MIG	95	NIQ.	
	Phe	Ser	Ala	Ala		Gly	Ser	Pro	Tle		Glu	I.ve	Aen	Δ1 =		λ1 <del>-</del> 3	
45				100		013			105		Olu	Буз	nsp	110	FLO	Ala	
	Lev	His	Lvs		Leu	Thr	Asn	Met			Asp	Alσ	Glv		Len	Δla	
			115					120		~		•••	125	713 P	De u	irrer '	
	Thr	Arg		Ala	Lvs	Glu	Lvs		Met	Ara	Ile	Ara		Phe	Ala	Phe	
		-	_	-		-	-				_						

		130					135					140					
	145					150					155	Asp	Lys			Lys 160	
5					165					170					1.75	Ala	
		Val		180					185					190		_	· X*
10			195					200					205			Trp	
		Ser 210		•			215					220					
	Thr 225	Leu	His	Thr	Asn	Pro 230	Ala	Phe	Gln	Gln	Gln 235		Gln	Lys	Ala	Lys 240	
15	Asp	Glu	Phe	Ala	Lys 245	Gln	Gln	Lys									
	IN	FORM									•						
? <b>0</b>		(i)	( <i>I</i> (E	A) LE 3) TY C) ST	ENGTI PE: PRANI	HARAG H: nucl DEDNI DGY:	735 l leic ESS:	oase acio doul	pai: d	rs			·.				
?5		(iii) (iv)	MOI HYI ANI ORI	LECUI POTHE CI-SE GINA	ETICA ENSE: L SO	PE: AL: 1 : NO DURCE	gend 10 E:	omic	DNA a fic	i	_						
30		(ix)	(E FEA	3) SI LTURE	'RAIN ::	J: I <i>I</i>	AM 13		1 11(	-ar re	<b>a</b>			•			
	(vi)	SE.	(E	3) LC	CATI	CEY:	17										
15	NTC	SEC	2 2 2 2 (OE:MC	יב הבי	OCK1	PTIC	)N: S	SEQ .	א מו	):27:	:						
	Met 1	AAA Lys	Lys	Ile	Leu 5	Leu	Ala	Thr	Leu	Ser 10	Cys	GCC Ala	GCG Ala	TTG Leu	ACG Thr 15	CAG Gln	48
	TTT	TCC	TTT	GCC	GCC	AAA	GAT	GTC	ACT	ACC	CAC	ССТ	GAG	CTT	ידמיד	արգուր	96
0	Phe	Ser	Phe	Ala 20	Ala	Lys	Asp	Val	Thr 25	Thr	His	Pro	Glu	Val	Tyr	Phe	96
	CTG	CAA	GAA	TCA	CAG	TCC	ATC	GAC	AGC	CTG	GCA	СТА	TTG	CCG	cce	CCG	144
	Leu	GIN	35	Ser	Gin	Ser	Ile	Asp 40	Ser	Leu	Ala	Leu	Leu 45	Pro	Pro	Pro	144
	CCG.	GCG	ATG	GAC	AGC	ATT	GAT	TTC	CTG	AAT	GAC	AAA	GCG	CAA	TAC	GAC	192
. <b>5</b>	Pro	Ala 50	Met	Asp	Ser	Ile	Asp 55	Phe	Leu	Asn	Asp	Lys 60	Ala	Gln	Tyr	Asp	
	GCC	GGG	AAA	ATA	GTG	CGC	AAT	ACT	CCG	CGT	GGC	AAG	CAG	GCT	TAT	GAT	240
	Ala	Gly	Lys	Ile	Val	Arq	Asn	Thr	Pro	Arσ	Glv	Lvs	Gln	Δla	Tur	n en	

	65					70					75					80	
	GAC	GCC	CAC	GTT	GCC	GGG	GAC	GGC	GTT	GCC	GCC	GCA	<b>ጉ</b> ጥጥ	TCC	AAC	GCC	288
5	Asp	Ala	His	Val	Ala 85	Gly	Asp	Gly	Val	Ala 90	Ala	Ala	Phe	Ser	Asn 95	Ala	200
	TTC	GGC	CTA	GAA	ATA	GCC	CAA	CGG	AAA	ACG	CCG	GAG	CTG	ттт	AAG	CTG	336
	Phe	GIY	Leu	Glu 100	Ile	Ala	Gln	Arg	Lys 105	Thr	Pro	Glu	Leu	Phe	Lys	Leu	330
	GTG	ATG	AAA	ATG	CGT	GAA	GAC	GCC	GĞC	GAT	TTG	GCG	ACC	CGC	AGC	GCC	384
10	vai	Met	Lys 115	Met	Arg	Glu	Asp	Ala 120	Gly	Asp	Leu	Ala	Thr 125	Arg	Ser	Ala	30.
	AAA	AAT	CAC	TAT	ATG	CGC	ATT	CGC	CCC	TTT	GCG	TTT	TAT	AAC	GAA	GCG	432
	гÀг	130	His	Tyr	Met	Arg	Ile 135	Arg	Pro	Phe	Ala	Phe 140	Tyr	Asn	Jlu	Ala	132
15	ACC	TGC	CGA	CCG	GAC	GAA	GAA	AGC	ACC	CTG	TCG	AAG	AAC	GGT	TCT	TAC	480
	inr	Cys	Arg	Pro	Asp	Glu	Glu	Ser	Thr	Leu	Ser	Lys	Asn	Gly	Ser	Tvr	100
	145					150					155					160	
	CCT	TCC	GGC	CAT	ACC	ACC	ATC	GGC	TGG	GCG	ACC	GCG	CTG	GTG	CTG	GCT	528
	Pro	Ser	Gly	His	Thr	Thr	Ile	Gly	Trp	Ala	Thr	Ala	Leu	Val	Leu	Ala	
20					165					170					175		
	GAA	ATC	AAC	CCC	GCC	AGG	CAG	GGT	GAA	ATC	CTG	CAG	CGC	GGC	тдт	GAT	576
25	Glu	Ile	Asn	Pro 180	Ala	Arg	Gln	Gly	Glu 185	Ile	Leu	Gln	Arg	Gly	Tyr	Asp	376
20	ATG	GGC	CAA		CGG	GTT	ATC	TGC		ጥልጥ	CAC	TCC	C 7 7	190	C 1 C		
	Met	Gly	Gln	Ser	Ara	Val	Ile	Cvs	Glv	Tur	Hie	Trn	CIn	AGC	GAC	GTG	624
			192					200					205				
	ACT	GCG	GCG	CGC	ATG	GCG	GCG	TCG	GCC	ATG	GTG	GCG	CGT	TTG	CAT	GCC	672
30	Inr	210	Ala	Arg	Met	Ala	Ala 215	Ser	Ala	Met	Val	Ala 220	Arg	Leu	His	Ala	
	GAA	CCC	ACC	TTC	GCC	GCC	CAG	CTG	CAA	AAG	GCC	AAA	GAC	GAA	TTC	AAC	720
	GIU	Pro	Thr	Phe	Ala	Ala	Gln	Leu	Gln	Lys	Ala	Lys	Asp	Glu	Phe	Asn	
	223					230					235		-			240	
35		CTG			TAA												735
	GIÀ	Leu	гÀг	Lys													
	INF	ORMA															
			SEC	UENC	E CH	ARAC	TERI	STIC									
40			( <u>A</u>	L) LE	NGTH	: 24	4 am	ino	acid	ls							

- (B) TYPE: amino acid
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Serratia ficaria
  - (B) STRAIN: IAM 13540
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Met Lys Lys Ile Leu Leu Ala Thr Leu Ser Cys Ala Ala Leu Thr Gln

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	1				5					10					15		
	Phe	Ser	Phe	Ala 20	Ala	Lys	Asp	Val	Thr 25	Thr	His	Pro	Glu	Val 30	Tyr	Phe	
5	Leu	Gln	Glu 35	Ser	Gln	Ser	Ile	Asp 40	Ser	Leu	Ala	Leu	Leu 45	Pro	Pro	Pro	
	Pro	Ala 50	Met	Asp	Ser	Ile	Asp 55	Phe	Leu	Asn	Asp	Lys 60		Gln	Tyr	Asp	
10	Ala 65	Gly	Lys	Ile	Val	Arg 70	Asn	Thr	Pro	Arg	Gly 75		Gln	Ala	Tyr	Asp 80	
	Asp _.	Ala	His	Val	Ala 85	Gly	Asp	Gly	Val	Ala 90		Ala	Phe	Ser	Asn 95	Ala	
	Phe	Gly	Leu	Glu 100	Ile	Ala	Gln	Arg	Lys 105	Thr	Pro	Glu	Leu	Phe 110	Lys	Leu	
15	Val	Met	Lys 115	Met	Arg	Glu	Asp	Ala 120	Gly	Asp	Leu	Ala	Thr 125	Arg	Ser	Ala	
	Lys	Asn 130	His	Tyr	Met	Arg	Ile 135	Arg	Pro	Phe	Ala	Phe 140	Tyr	Asn	Glu	Ala	
20	Thr 145	Суѕ	Arg	Pro	Asp	Glu 150	Glu	Ser	Thr	Leu	Ser 155		Asn	Gly		Tyr 160	
	Pro	Ser	Gly	His	Thr 165	Thr	Ile	Gly	Trp	Ala 170		Ala	Leu	Val	Leu 175	Ala	
	Glu	Ile	Asn	Pro 180	Ala	Arg	Gln	Gly	Glu 185		Leu	Gln	Arg	Gly 190	Tyr	Asp	
25	Met	Gly	Gln 195	Ser	Arg	Val	Ile	Cys 200		Tyr	His	Trp	Gln 205	Ser	Asp	Val	
	Thr	Ala 210	Ala	Arg	Met	Ala	Ala 215	Ser	Ala	Met	Val	Ala 220	Arg	Leu	His	Ala	
30	225		Thr		Ala	Ala 230	Gln	Leu	Gln	Lys	Ala 235	Lys	Asp	Glu	Phe	Asn 240	
			Lys														
	INFO		TION SEC	UENC	E CH	IARAC	TERI	STIC									
35			(E	A) LE B) TY	PE:	nucl	eic	acid		5							
			( [	) 51 ) TO	POLC	GY:	line	ar									
40	(	(iii)	MOI HYP	OTHE	TICA	⊥: N	othe O	r DN	JAs	ynth	etic	DNA					
		(xi)	ANT	UENC	EDE	SCRI	PTIC	N: S	EQ I	D NC	:29:						
			CA C						•								21
45	INFC		TION SEC	UENC	E CH	ARAC	TERI	STIC									
•			(A	L) LE	NGTH PE:	: 2 nucl	l ba eic	se p	airs I								
50				•													
				C) ST					gle								
e e			) MO:	LECUI	LE TY	PE:	othe		۱As	ynth	netic	: DNA					
55			) AN' ) SE(				PTIC	ON: S	SEQ ]	ID NO	):30:						
	GCC			GCAT(					_								21

#### SEQUENCE LISTING

5	(1) GENE	RAL INFORMATION:	
	(i)	APPLICANT:	
		(A) NAME: Ajinomoto Co., Inc.	
10		(B) STREET: No.15-1, Kyobashi 1-chome, Chuo-ku	
		(C) CITY: Tokyo	
		(E) COUNTRY: Japan	
		(F) POSTAL CODE (ZIP): 104 Japan	
15		•	
	(ii)	TITLE OF INVENTION: Method for producing nucleoside-5'-phosphate ester	е
20	(iii)	NUMBER OF SEQUENCES: 30	
	(iv)	COMPUTER READABLE FORM:	
		(A) MEDIUM TYPE: Floppy disk	
25		(B) COMPUTER: IBM PC compatible	
		(C) OPERATING SYSTEM: PC-DOS/MS-DOS	
		(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)	
30	(v)	CURRENT APPLICATION DATA:	
		APPLICATION NUMBER: EP 97309365.1	
	INFORMAT	CION FOR SEQ ID NO:1:	
35	(i)	SEQUENCE CHARACTERISTICS:	
		(A) LENGTH: 20 amino acids	
		(B) TYPE: amino acid	
		(D) TOPOLOGY: linear	
40	(ii)	MOLECULE TYPE: peptide	
	(V)	PROTEIN FRAGMENT TYPE: N-terminal	
	(Vi)	ORIGINAL SOURCE:	
		(A) ORGANISM: Morganella morganii	
45		(B) STRAIN: NCIMB 10466	
	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:1:	
	Ala Ile P	ro Ala Gly Asn Asp Ala Thr Thr Lys Pro Asp Leu Tyr Tyr	
	1	5 10 15	
50	Leu Lys A		
		20	

	IN	FORM	ATIO	N FO	R SE	QID	NO:	2:									
		(i	) SE	QUEN	CE C	HARA	CTER	ISTI	cs:								•
5			(2	A) L	ENGT	H: '	750	base	pai	rs							
			(1	B) T	YPE:	nuc	leic	aci	d.								
			(	c) s	TRAN	DEDN	ESS:	dou	ble								
			(1	D) T	OPOL	OGY:	lin	ear									
10		(ii	) MO	LECU	LE T	YPE:	gen	omic	DNA								
		(iii	HY	POTH	ETIC	AL: 1	ОИ										
		(iv	AN:	ri-si	ENSE	: NO											
		(vi	OR	IGIN	AL S	OURC	E:										
15			(2	A) O	RGAN	ISM:	Mor	gane	lla :	morg	anii						
			(1	B) S'	TRA I	N: N	CIMB	104	66								
		(ix	) FE	ATUR	E:									•			
			(7	A) N	AME/	KĘY:	CDS										
20			(1	B) L	OCAT:	ION:	1	747									
		(ix	) FE	ATUR	E:												
			(2	A) N	AME/I	KEY:	sig	_pep	tide								
			(1	B) L	OCAT:	ION:	1	60									
25		(ix	) FE	ATUR	E :												
			(1	A) N	AME/I	KEY:	mat_	pept:	ide			-					
			(1	B) L	CAT:	ION:	61.,	747									
	(xi	) SE(	QUEN	CE DI	ESCR	IPTIC	: NC	SEQ	ID N	0:2:							
30	ATG	AAG	AAG	AAT	ATT	ATC	GCC	GGT	TGT	CTG	TTC	TCA	CTG	TTT	TCC	CTT	48
	Met	Lys	Lys	Asn	Ile	Ile	Ala	Gly	Cys	Leu	Phe	Ser	Leu	Phe	Ser	Leu	
	-20					-15					-10					-5	
	TCC	GCG	CTG	GCC	GCG	ATC	CCG	GCG	GGC	AAC	GAT	GCC	ACC	ACC	AAG	CCG	96
3 <b>5</b>	Ser	Ala	Leu	Ala	Ala	Ile	Pro	Ala	Gly	Asn	Asp	Ala	Thr	Thr	Lys	Pro	
					1				5					10			
	GAT	TTA	TAT	TAT	CTG	AAA	AAT	GAA	CAG	GCT	ATC	GAC	AGC	CTG	AAA	CTG	144
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Glu	Gln	Ala	Ile	Asp	Ser	Leu	Lys	Leu	
40			15					20					25				
	TTA	CCG	CCA	CCG	CCG	GAA	GTC	GGC	AGT	ATT	CAG	TTT	TTA	AAT	GAT	CAG	192
	Leu	Pro	Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Gln	Phe	Leu	Asn	Asp	Gln	
		30					35					40					
45	GCA	ATG	TAT	GAG	AAA	GGC	CGT	ATG	CTG	CGC	AAT	ACC	GAG	CGC	GGA	AAA	240
	Ala	Met	Tyr	Glu	Lys	Gly	Arg	Met	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Lys	
	45					50					55					60	
	CAG	GCA	CAG	GCA	GAT	GCT	GAC	CTG	GCC	GCA	GGG	GGT	GŢG	GCA	ACC	GCA	288
50	Gln	Ala	Gln	Ala	Asp	Ala	Asp	Leu	Ala	Ala	Gly	Gly	Val	Ala	Thr	Ala	
					65					70					75		
	TTT	TCA	GGG	GCA	TTC	GGC	TAT	CCG	ATA	ACC	GAA	AAA	GAC	TCT	CCG	GAG	336
	Phe	Ser	Gly	Ala	Phe	Gly	Tyr	Pro	Ile	Thr	Glu	Lys	Asp	Ser	Pro	Glu	
55				80					85					90			

	CTG	TAT	AAA	CTG	CTG	ACC	AAT	ATG	ATT	GAG	GAT	GCC	GGT	GAT	CTT	GCC	384
	Leu	Tyr	Lys	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala	
_			95					100					105				
5	ACC	CGC	TCC	GCC	AAA	GAA	CAT	TAC	ATG	CGC	ATC	CGG	CCG	TTT	GCG	TTT	432
	Thr	Arg	Ser	Ala	Lys	Glu	His	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	
		110					115					120					
	TAC	GGC	ACA	GAA	ACC	TGT	AAT	ACC	AAA	GAT	CAG	AAA	AAA	CTC	TCC	ACC	480
10	Tyr	Gly	Thr	Glu	Thr	Сув	Asn	Thr	Lys	Asp	Gln	Lys	Lys	Leu	Ser	Thr	
	125					130					135					140	
	AAC	GGA	TCT	TAC	CCG	TCA	GGT	CAT	ACG	TCT	ATC	GGC	TGG	GCA	ACC	GCA	528
45	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala ·	
15					145					150					155		
	CTG	GTG	CTG	GCG	GAA	GTG	AAC	CCG	GCA	AAT	CAG	GAT	GCG	ATT	CTG	GAA	576
	Leu	Val	Leu	Ala	Glu	Val	Asn	Pro	Ala	Asn	Gln	Asp	Ala	Ile	Leu	Glu	
				160					165					170			
20	CGG	GGT	TAT	CAG	CTC	GGA	CAG	AGC	CGG	GTG	ATT	TGC	GGC	TAT	CAC	TGG	624
	Arg	Gly	_	Gln	Leu	Gly	Gln	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	
			175					180					185				
					GAT												672
25	Gln		Asp	Val	Asp	Ala		Arg	Ile	Val	Gly		Ala	Ala	Val	Ala	
		190					195					200					
					GAT												720
		Leu	His	Ser	Asp		Ala	Phe	Gln	Ala		Leu	Ala	Lys	Ala	•	
30	205					210					215					220	
					CAA					TAA							750
	GIN	GIU	Phe	Ala	Gln	rys	ser	GIN	_								
					225				229								
35	TNI	20011		I FOI		. TD	NO.										
	11/1				R SEÇ CE CI	_			70.								
		( = )			ENGT:					10							
			•	•	PE:				4010	10							
40					POL												
		(55)	•	•	E TY												
					AL SC												
		( • - )			RGANI			ranel	lar	noras	mii						
45					RAIN					org							
			,-	.,													
		(xi)	SEC	UENC	E DE	SCRI	PTIC	ON: S	SEO I	D NO	):3:				,		
	Met				Ile							Ser	Leu	Phe	Ser	Leu	
50	-20	-•-	-•-			-15		•			-10					-5	
		Ala	Leu	Ala	Ala	Ile	Pro	Ala	Gly	Asn		Ala	Thr	Thr	Lys	_	
					1				5		•		_	10			
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Glu	Gln	Ala	Ile	Asp	Ser		Lys	Leu	
<i>55</i>	-		15	_		-		20				•	25		- 4 -		

	204		110			314	AGI	GIA	261	116	GIH	File	reu	ASD	ASP	GII
		30					35					40				
	Ala	Met	Tyr	Glu	Lys	Gly	Arg	Met	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Lya
5	45					50					55				•	60
	Gln	Ala	Gln	Ala	Asp	Ala	Asp	Leu	Ala	Ala	Gly	Gly	Val	Ala	Thr	Ala
					65					70					75	
	Phe	Ser	Gly	Ala	Phe	Gly	Tyr	Pro	Ile	Thr	Glu	Lys	Asp	Ser	Pro	Glu
10				80					85			_		90		
	Leu	Tyr	Lys	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala
			95					100					105	_		
	Thr	Arg	Ser	Ala	Lys	Glu	His	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe
15	•	110					115					120				
	Tyr	Gly	Thr	Glu	Thr	Cys	Asn	Thr	Lys	Asp	Gln	Lys	Lys	Leu	Ser	Thr
	125					130					135	-	-			140
	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	İle	Glv	Tro	Ala	Thr	
20		_		-	145		-			150		•			155	
	Leu	Val	Leu	Ala	Glu	Val	Asn	Pro	Ala	Asn	Gln	asp	Ala	Ile		
				160					165			•		170		
	Arg	Gly	Tyr	Gln	Leu	Gly	Gln	Ser	Arg	Val	Ile	Сув	Glv			Tro
25	_	_	175			-		180				•	185	-1-		F
	Gln	Ser	Asp	Val	Asp	Ala	Ala	Arg	Ile	Val	Glv	Ser		Ala	Val	Ala
		190	•		•		195				1	200				
	Thr	Leu	His	Ser	Asp	Pro			Gln	Ala	Gln		Ala	T.vg	212	Lve
20	205					210					215			<b>D</b> , 0	n.a	220
30		Glu	Phe	Ala	Gln		Ser	Gln	Lvs							220
					225	-1-		••••	229							
	INI	FORM	OITA	1 FOF	SEC	ID	NO: 4	1:								
35				QUENC		-			cs:							
				A) LE						is						
				3) TY							•					
			•	, ) TC												
10		(ii)	•	ECUL												
	•			GINA			-									
		( - /		A) OR				anel	lam	orga	nii					
				3) ST			_			ior gu						
15		(xi)		UENC						ם אכ						
	Ala	Ile										Pro	Agn	T 011	T	
	1				5					10	-10	110	veħ	Leu	15	TYE
	<del>-</del>	Lys	Asn	Glu	_	Ala	Tle	Asn	Ser		Lva	Len	Len	Pro		D
50		-1-		20	<b></b>			p	25	Dea	Dys	Leu	Leu	30	PIO	Pro
	Pro	Glu	Va 1		Sor	Tlo	Gln	Dho		A ca	7.55	C1-	n 1 -		<b></b>	۵١
	110	J_4	35	JLY	J-1	116	3111	40	Tea	VOII	veħ	GTU		net	ryr	GIU
	Luc	Gly		Met	ī.eu	Ara	Δan		Gl	A	G1	T	45	<b>31</b> -	<b>~</b> 1	
55	nya	50	nr y	.766	<b>J</b> ∈u	nrg		THE	GIU	urd	GIÀ		GIN	ATA	GIN	Ala
		30					55					60				

		Ala	Asp	Leu	Ala	Ala	Gly	Gly	Val	Ala	Thr	Ala	Phe	Ser	Gly	Ala
	65					70					75					80
5	Phe	Gly	Tyr	Pro		Thr	Glu	Lys	yab		Pro	Glu	Leu	Tyr	Lys	Leu
	Teu	Th-	a a n	Mot	85	C1	N	21-	<b>63</b>	90				_	95	
	Deu	1111	Asn	100	116	GIU	мвр	wra	105	Авр	Leu	Ala	Thr		Ser	Ala
	Lvs	Glu	His		Met	Ara	Tle	Ara		Phe	A1 =	Phe	Tur	110	Th-	G1
10	-,-		115	-1-		••••		120		rne	vre	FIIC	125	GIY	Int	GIU
	Thr	Сув	Asn	Thr	Lys	Asp	Gln		Lys	Leu	Ser	Thr		Glv	Ser	Tvr
		130			_	_	135	-	•			140		1		-,-
	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	Leu	Val	Leu	Ala
15	145					150					155					160
	Glu	Val	Asn	Pro	Ala	Asn	Gln	Asp	Ala	Ile	Leu	Glu	Arg	Gly	Tyr	Gln
					165					170					175	
20	Leu	Gly	Gln		Arg	Val	Ile	Cys		Tyr	His	Trp	Gln	Ser	Asp	Val
20				180			_,	_	185					190		
	Asp	ATA	Ala 195	Arg	116	Val	GIÅ		Ala	Ala	Val	Ala		Leu	His	Ser
	Asn	Pro	Ala	Phe	Gln	Al a	Gln	200	Al n	Tuá	21.	· T	205	<b>~</b> 1	<b>D</b> b	• • •
25	пор	210		1	<b>G1</b> 11	ura	215	Leu	VIG	гуя	WIG	220	GIN	GIU	Pne	Ala
	Glr		Ser	Gln	Lys	3						220				
	225				229											
30	INF	FORMA	TION	FOR	SEC	] ID	NO: 9	<b>:</b>								
		(i)	SEC	UENC	E CH	IARAC	TERI	STIC	s:							
			-	) LE					cids	;						
				TY		•										
35		/::>	MOL	) TO												
		(v)		OTEI					N_+	~~~ i	m n 1					
			ORI						N-C	CTMT	naı					
40		, , ,		) OR				eric	hia	blat	tae					
				) ST												
		(xi)	SEQ	UENC	E DE	SCRI	PTIO	N: S	EQ Į	D NO	:5:					
	Leu											Thr	Lys	Pro	Asp	Leu
45	1				5					10					15	
							•									
	INF		TION													
		(1)	SEQ													
50			•	) LE ) TY				ase	-	8						
				) ST												
				) TO			· .									
<i>55</i>		(ii)	MOL	-					DNA							
			HYP													

		(iv	) AN	TI-S	Ense	: NO											
		(vi	OR	IGIN.	AL S	OURC	E: '					•					
_			(4	A) O	RGAN	ISM:	Esc	heri	chia	bla	ttae						
5			(	B) S	TRAI	N: J	CM 1	650									
		(ix	) FE	ATUR	E:												
			(2	A) N	AME/	KEY:	CDS										
			(1	B) L	OCAT	ION:	1	747									•
10		(ix)	) FE	ATUR	E:												
			(2	A) N.	AME/	KEY:	sig	_pep	tide								
			(1	B) L	OCAT	ION:	1	54									
15		(ix	) FE	ATUR	E:												
15			(2	A) N	AME/	KEY:	mat_	pept	ide								
			(1	B) L	OCAT	ion:	55	747									
	(xi	) SE(	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:6:							
20	ATG	AAA	AAA	CGT	GTT	CTG	GCA	GTT	TGT	TTT	GCC	GCA	TTG	TTC	TCT	TCT	48
20	Met	Lys	Lys	Arg	Val	Leu	Ala	Val	Сув	Phe	Ala	Ala	Leu	Phe	Ser	Ser	
	-18			-15					-10					-5			
	CAG	GCC	CTG	GCG	CTG	GTC	GCT	ACC	GGC	AAC	GAC	ACT	ACC	ACG	AAA	CCG	96
25	Gln	Ala	Leu	Ala	Leu	Val	Ala	Thr	Gly	Asn	Asp	Thr	Thr	Thr	Lys	Pro	
25			1				5					10			_		
	GAT	CTC	TAC	TAC	CTC	AAG	AAC	AGT	GAA	GCC	ATT	AAC	AGC	CTG	GCG	CTG	144
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ser	Glu	Ala	Ile	Asn	Ser	Leu	Ala	Leu	
00	15					20					25					30	
30	TTG	CCG	CCA	CCA	CCG	GCG	GTG	GGC	TCC	ATT	GCG	TTT	CTC	AAC	GAT	CAG	192
	Leu	Pro	Pro	Pro	Pro	Ala	Val	Gly	Ser	Ile	Ala	Phe	Leu	Asn	Asp	Gln	
					35					40					45		
35	GCC	ATG	TAT	GAA	CAG	GGG	CGC	CTG	CTG	CGC	AAC	ACC	GAA	CGC	GGT	AAG	240
35	Ala	Met	Tyr	Glu	Gln	Gly	Arg	Leu	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Lys	
				50					55					60			
	CTG	GCG	GCG	GAA	GAT	GCA	AAC	CTG	AGC	AGT	GGC	GGG	GTG	GCG	AAT	GCT	288
40	Leu	Ala	Ala	Glu	Asp	Ala	Asn	Leu	Ser	Ser	Gly	Gly	Val	Ala	Asn	Ala	
40			65	5				70	ס				7	5			
	TTC	TCC	GGC	GCG	TTT	GGT	AGC	CCG	ATC	ACC	GAA	AAA	GAC	GCC	CCG	GCG	336
	Phe	Ser	Gly	Ala	Phe	Gly	Ser	Pro	Ile	Thr	Glu	Lys	Asp	Ala	Pro	Ala	
45		80					85					90					
40	CTG	CAT	AAA	TTA	CTG	ACC	AAT	ATG	ATT	GAG	GAC	GCC	GGG	GAT	CTG	GCG	384
	Leu	His	Lys	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala	
	95					100			•		105					110	
50	ACC	CGC	AGC	GCG	AAA	GAT	CAC	TAT	ATG	CGC	ATT	CGT	CCG	TTC	GCG	TTT	432
30	Thr	Arg	Ser	Ala	Lys	Asp	His	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	
					115					120					125		
	TAT	GGG	GTC	TCT	ACC	TGT	AAT	ACC	ACC	GAG	CAG	GAC	AAA	CTG	TCC	AAA	480
55	Tyr	Gly	Val	Ser	Thr	Сув	Asn	Thr	Thr	Glu	Gln	Asp	Lys	Leu	Ser	Lys	
				130					135					140		-	

	WWI	GGC	101	INI	CCG	100	GGG	CAI	ACC	TCT	ATC	GGC	TGG	GCT	ACT	GCG	528
	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	
			145					150					155				
5	CTG	GTG	CTG	GCA	GAG	ATC	AAC	CCT	CAG	CGC	CAG	AAC	GAG	ATC	CTG	AAA	576
	Leu	Val	Leu	Ala	Glu	Ile	Asn	Pro	Gln	Arg	Gln	Asn	Glu	Ile	Leu	Lys	
		160					165					170					
	CGC	GGT	TAT	GAG	CTG	GGC	CAG	AGC	CGG	GTG	ATT	TGC	GGC	TAC	CAC	TGG	624
10	Arg	Gly	Tyr	Glu	Leu	Gly	Gln	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	
	175					180					185					190	
				GTG													672
	Gln	Ser	Asp	Val	Asp	Ala	Ala	Arg	Val	Val	Gly	Ser	Ala	Val	Val	Ala	
15					195					200					205		
				ACC													720
	Thr	Leu	His	Thr	Asn	Pro	Ala	Phe	Gln	Gln	Gln	Leu	Gln	Lys	Ala	Lys	
				210					215					220			
20				GCC						TAA							750
	Ala	Glu		Ala	Gln	His	Gln		Lys								
			225					230									
								_									
25	INI			N FOI		_											
		(i)		QUEN						_							
			•	A) LI					acio	ls							
			•	B) T													
30			•	) T	•												
		· .		LECUI			-	cein									
		(VI)		IGIN						<b>-</b> 2 - 4							
		•	•	A) OF					cnia	ртат	tae						
35		(***	-	3) SI QUENC					reo :	TD NO							
	Wet			Arg								71-	T 0	Dha	C	C	
	-18	2y5	₽y 5	-15	491	Deu	NIG.	Val	-10	File	ALA	wra	rea	-5	Ser	ser	
•		Als	T.o.u	Ala	Leu	Va 1	Als	Th-		y an	N.c.	mb	mh		T	D	
40	<b>01</b>		1		200	•••	5	* * * * *	GIJ	non	veb	10	1111	THE	гув	Pro	
	Asp	Leu		Tyr	Leu	Lvs		Ser	Glu	Ala	Tle		Ser	T.ou	A 1 =	Len	
	15		-1-	-1-		20			<b>414</b>		25	7.011	261	Leu	nia	30	
		Pro	Pro	Pro	Pro		Val	Glv	Ser	Tle		Dha	T 0	Aan	N.a.s	-	
45					35		142	01,	501	40	nta	riic	Deu	non	45	GIII	
+5	Ala	Met	Tvr	Glu		G) v	Ara	Leu	Leu		Aan	Thr	Glu	Ara		Tres	
			-7-	50		,	•••		55				GIU	60	GIA	Lys	
	Leu	Ala	Ala	Glu	Asp	Ala	Asn	Leu		Ser	Glv	Glv	Va l		D 0 10	מומ	
			65				•••••	70		DC1	OLJ	Gly	75		VOII	NIG	
50	Phe	Ser		Ala	Phe	Glv	Ser			Thr	Glu	ī.ve			Pro	בומ	
		80				,	85				~~u	90	op	.,,6	210	11 th	
-	Leu		Lvs	Leu	Leu	Thr		Met	Ile	Glu	Agn		Glv	Agn	٠٠٩.٦	Ala	
	95		- 4 -			100					105		1		J∉ u	110	
55	-															110	

	Thr	Arg	Ser	Ala	Lys 115	Asp	His	Tyr	Met	Arg 120	Ile	Arg	Pro	Phe	Ala 125	Phe
5	Tyr	Gly	Val	Ser 130	Thr	Сув	Asn	Thr	Thr 135	Glu	Gln	Asp	Lув	Leu 140	Ser	Lys
	Asn	Gly	Ser 145		Pro	Ser	Gly	His 150		Ser	Ile	Gly	Trp 155	Ala	Thr	Ala
10	Leu	Val		Ala	Glu	Ile	Asn 165	Pro	Gln	Arg	Gln	Asn 170		Ile	Leu	Lys
	Arg 175	Gly	Tyr	Glu	Leu	Gly 180	Gln	Ser	Arg	Val	Ile 185	Сув	Gly	Tyr	His	Trp 190
15	Gln	Ser	Asp	Val	Asp 195	Ala	Ala	Arg	Val	Val 200	Gly	Ser	Ala	Val	Val 205	Ala
	Thr	Leu	His	Thr 210	Asn	Pro	Ala	Phe	Gln 215	Gln	Gln	Leu	Gln	Lys 220	Ala	Lys
20	Ala	Glu	Phe 225	Ala	Gln	His	Gln	<b>Lys</b> 230	Lys							
	INI	FORM	10ITA	ı FOI	R SEÇ	2 ID	ио: 8	3:								
25		(i)		_	CE CE Engti					is	r					
			•	•	PE:										•	
30					LE TY		-	cein						•		
30		<b>(</b> )	(2	A) OI	rgani rrain	SM:	Esci		hia	blat	tae			,		
		(xi)			CE DE				SEQ 1	ED NO	:8:					
35	Leu 1	Ala	Leu	Val	Ala 5	Thr	Gly	Asn	Asp	Thr 10	Thr	Thr	Lys	Pro	Asp 15	Leu
	Tyr	Tyr	Leu	Lys 20	Asn	Ser	Glu	Ala	Ile 25	Asn	Ser	Leu	Ala	Leu 30	Leu	Pro
40	Pro	Pro	Pro 35	Ala	Val	Gly	Ser	Ile 40	Ala	Phe	Leu	Asn	Авр 45	Gln	Ala	Met
	Tyr	Glu 50	Gln	Gly	Arg	Leu	Leu 55	Arg	Asn	Thr	Glu	Arg 60	Gly	Lys	Leu	Ala
45	Ala 65	Glu	Авр	Ala	Asn	Leu 70	Ser	Ser	Gly	Gly	<b>Val</b> 75	Ala	Asn	Ala	Phe	Ser 80
	Gly	Ala	Phe	Gly	Ser 85	Pro	Ile	Thr	Glu	Lys 90	Хвр	Ala	Pro	Ala	Leu 95	His
50 .	Lys	Leu	Leu	Thr 100	Asn	Met	Ile	Glu	Asp 105	Ala	Gly	Двр	Leu	Ala 110	Thr	Arg
	Ser	Ala	Lys 115	Asp	His	Tyr	Met	Arg 120	Ile	Arg	Pro	Phe	Ala 125	Phe	Tyr	Gly
55	Val	Ser 130	Thr	Cys	Asn	Thr	Thr 135	Glu	Gln	Asp	Lys	Leu 140	Ser	Lys	Asn	Gly

	261	TAT	PLU	261	GIA	UIB	The	ser	116	GTA	Trp	Ala	Thr	Ala	Leu	Val	
	145					150					155					160	
_	Leu	Ala	Glu	Ile	Asn	Pro	Gln	Arg	Gln	Asn	Glu	Ile	Leu	Lys	Arg	Gly	
5					165					170				•	175	•	
	Tyr	Glu	Leu	Gly	Gln	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	Gln	Ser	
				180					185	_	·	•		190			
	Asp	Val	Asp	Ala	Ala	Arg	Val	Val	Gly	Ser	Ala	Val	Val	Ala	Thr	Leu	
10			195					200	-				205				
	His	Thr	Asn	Pro	Ala	Phe	Gln	Gln	Gln	Leu	Gln	Lys	Ala	Lvs	Ala	Glu	
		210					215					220		•			
	Phe	Ala	Gln	His	Gln	Lys	Lys										
15	225					230											
	IN	FORM	ATIO	N FOI	R SE	Q ID	NO:	9:					,				
		( <u>1</u> )	) SE	QUEN	CE CI	IARA	TER:	ISTIC	cs:								
20			(2	A) LI	ENGT	ł: 2	20 b	ase p	paire	3							
			(1	B) T	PE:	nucl	leic	acio	i								
			((	C) 53	<b>CRANI</b>	DEDNE	ess:	sing	gle								
			(1	D) T	OPOLO	χς:	line	ear								•	
25		(ii)	MOI	LECUI	LE TY	PE:	othe	er Di	Ι <b>Α.</b> .ε	yntl	retio	DN1	A				
	(	(iii	HYI	POTHE	etic/	L: N	10										
		(iv)	) ANT	ri-se	ENSE:	NO											
		(xi	) SEÇ	QUENC	CE DE	SCRI	PTIC	ON: S	EQ 1	D NO	9:						
30	CCT	CGAGO	TC (	BACGO	TAT	:G											20
	INE			V FOR													
		(i)		DUENC													
35				A) LE				•		3							
			-	3) TY													
				2) SI				_	le								
			-	) TC													
40				ECUI				er DN	Ае	ynth	etic	DNA	<b>L</b>				
	(			POTHE													
				I-SE													
				QUENC			PTIC	N: S	EQ I	D NO	:10:						
45	ATTC	GCCA	ICA 1	CGCC	ACTG	CT						•					21
	TNE	ODVA	m T O N	r ron	- CE-C		NO - 1	• .									
	INF			FOR					_								
		(1)		UENC													
50				) LE				_							•		
				) TY :) ST													
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		/ { { } } }		) TO					<b>x</b> -	4-1		D					
55	,			ECUL OTHE				אט ב	n8	ynth	etic	DNA	•				
	,	)	птР	OTUE	LICH	Ν .بد	J										

	(iv)	ANTI-SENSE: NO	
	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:11:	
5	TAGCCCAG	CC GGTAGAGGTA TG	22
	INFORMAT	ION FOR SEQ ID NO:12:	
10	(i)	SEQUENCE CHARACTERISTICS:	
		(A) LENGTH: 23 base pairs	
		(B) TYPE: nucleic acid	
		(C) STRANDEDNESS: single	
15		(D) TOPOLOGY: linear	
	(ii)	MOLECULE TYPE: other DNAsynthetic DNA	
	(iii)	HYPOTHETICAL: NO	
	(iv)	ANTI-SENSE: NO	
20	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:12:	
	TGCATCTG	CC TGCGCCTGCT TAC	23
	INFORMAT	ION FOR SEQ ID NO:13:	
25	(i)	SEQUENCE CHARACTERISTICS:	
		(A) LENGTH: 20 base pairs	
		(B) TYPE; nucleic acid	
30		(C) STRANDEDNESS: single	
30		(D) TOPOLOGY: linear	
	(ii)	MOLECULE TYPE: other DNAsynthetic DNA	
	(iii)	HYPOTHETICAL: NO	
35	(iv)	ANTI-SENSE: NO	
00	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:13:	
	AACGCGCC	GT AGAAAGCATT	20
	INFORMAT	ION FOR SEQ ID NO:14:	
40		SEQUENCE CHARACTERISTICS:	
		(A) LENGTH: 21 base pairs	
		(B) TYPE: nucleic acid	
		(C) STRANDEDNESS: single	
45		(D) TOPOLOGY: linear	
	(ii)	MOLECULE TYPE: other DNAsynthetic DNA	
		HYPOTHETICAL: NO	
50		ANTI-SENSE: NO	
50	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:14:	
	GTCCTGGT	CT TTGGTATTAC A	21

	INFORMATION FOR SEQ ID NO:15:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 26 base pairs	
5	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
10	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
	CACATCGCCA GCGGCCAGGT CTGCAT	26
15		
,,	INFORMATION FOR SEQ ID NO:16:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 21 base pairs	
	(B) TYPE: nucleic acid	
20	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
	(iii) HYPOTHETICAL: NO	
25	(iv) ANTI-SENSE: NO	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
	GCATATAGTG TTCTTTCGCG C	21
30	INFORMATION FOR SEQ ID NO:17:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 22 base pairs	
	(B) TYPE: nucleic acid	
35	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
	(iii) HYPOTHETICAL: NO	
40	(iv) ANTI-SENSE: NO	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
	ATTACAGGTT TCGACCCCAT AA	22
45	INFORMATION FOR SEQ ID NO:18:	
40	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 25 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
50	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	
55		

	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:	
	TGATGCATGT CCGGGCTGTC TTTTT	25
5		
	INFORMATION FOR SEQ ID NO:19:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 25 base pairs	
10	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
15	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: YES	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:	
	CTGGATCCTG TGGCTATCAT CACCT	25
20		
	INFORMATION FOR SEQ ID NO:20:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 25 base pairs	
25	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: other DNAsynthetic DNA	
30	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:	
	CTGGATCCGA CGCGATTTTA CCATA	25
35		
	INFORMATION FOR SEQ ID NO:21:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 747 base pairs	
40	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: genomic DNA	
45	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	,
	(vi) ORIGINAL SOURCE:	
	(A) ORGANISM: Providencia stuartii	
50	(B) STRAIN: ATCC 29851	
	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 1744	
55		

	(xi	) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:21	:						
	ATG	AAA	AAA	CTA	TTA	GCA	GTA	TTC	TGC	GCA	GGG	GCT	TTT	GTT	TCA	ACC	48
5	Met	Lys	Lys	Leu	Leu	Ala	Val	Phe	Сув	Ala	Gly	Ala	Phe	Val	Ser	Thr	
	1				5					10					15		
	AGT	GTA	TTT	GCG	GCG	ATC	CCT	CCC	GGC	AAT	GAT	GTG	ACA	ACT	AAA	CCC	96
	Ser	Val	Phe	Ala	Ala	Ile	Pro	Pro	Gly	Asn	Asp	Val	Thr	Thr	Lys	Pro	
10				20					25					30			
	GAT	CTT	TAT	TAT	TTA	AAA	AAC	TCA	CAG	GCT	ATT	GAT	AGT	TTA	GCG	TTA	144
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ser	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu	
			35					40					45				
15	TTG	CCG	CCA	CCA	CCT	GAA	GTG	GGC	AGT	ATC	TTA	TTT	TTA	AAC	GAC	CAA	192
	Leu	Pro	Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Leu	Phe	Leu	Asn	Asp	Gln	
		50					55					60					
	GCG	ATG	TAT	GAA	AAA	GGC	CGT	TTA	TTG	CGA	AAT	ACT	GAG	CGT	GGA	GAA	240
20	Ala	Met	Tyr	Glu	Lys	Gly	Arg	Leu	Leu	Arg	Asn	Thr	Glu	Arg	Gly	Glu	
	65					70					75					80	
	CAA	GCC	GCT	AAG	GAT	GCT	GAT	CTG	GCT	GCG	GGC	GGT	GTT	GCG	AAC	GCA	288
	Gln	Ala	Ala	Lys	Asp	Ala	Asp	Leu	Ala	Ala	Gly	Gly	Val	Ala	Asn	Ala	
25					85					90					95		
		TCT															336
	Phe	Ser	Glu	Ala	Phe	Gly	Tyr	Pro	Ile	Thr	Glu	Lys	Asp	Ala	Pro	Glu	
				100					105					110			
30		CAT															384
	Ile	His	Lys	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Asp	Leu	Ala	
			115					120					125				
		CGC															432
35	Thr	Arg	Ser	Ala	Lys	Glu		Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	
		130					135					140					
		GGT															480
		Gly	Val	Ala	Thr		Asn	Thr	Lys	Asp	Gln	Asp	Lys	Leu	Ser	Lys	
40	145					150					155					160	
		GGC															528
	Asn	Gly	Ser	Tyr		Ser	Gly	His	Thr		Ile	Gly	Trp	Ala	Ser	Ala	
45					165					170					175		
45		GTA															576
	Leu	Val	Leu		Glu	Ile	Asn	Pro		Asn	Gln	Asp	Lys		Leu	Lys	
				180					185					190			
50		GGT															624
30	Arg	Gly		Glu	Leu	Gly	Gln		Arg	Val	Ile	Cys	_	Tyr	His	Trp	
			195					200		_			205				
		AGT															672
55	Gln	Ser	Asp	val	Asp	Ala		Arg	Ile	Val	Ala		Gly	Ala	Val	Ala	
~ <del>~</del>		210					215					220					

				•													
					AAC												720
	Thr	Leu	His	Ser	Asn	Pro	Glu	Phe	Gln	Lys	Gln	Leu	Gln	Lys	Ala	Lys	
5	225					230					235					240	
•	GAC	GAA	TTT	GCT	AAA	CTG	AAA	AAA	TAG								747
	yab	Glu	Phe	Ala	ГÀв	Leu	Lys	Lys									
					245												
10																	
	INI				R SEC												
		(1)		_	CE CI Engti					_ د							
			•	•	PE:				acı	as							
15			•	•	OPOLO												
		(111	•	•	LE TY												
					AL SC		-										
		, /			RGANI			vide	ncia	stua	artii	L					
20			•	-	TRAIN							-					
		(xi)	) SE	QUENC	CE DE	SCR	[PTI	ON: S	SEQ :	ID NO	0:22:	:					
	Met				Leu								Phe	Val	Ser	Thr	
	1				5					10					15		
25	Ser	Val	Phe	Ala	Ala	Ile	Pro	Pro	Gly	Asn	Asp	Val	Thr	Thr	Lys	Pro	
				20					25					30			
	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ser	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu	
			35					40					45				
30	Leu		Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Leu	Phe	Leu	Asn	Asp	Gln	
		50		_			55					60					
		Met	Tyr	Glu	ГÀв		Arg	Leu	Leu	Arg		Thr	Glu	Arg	Gly		
	65			•		70		•			75				_	80	
35	GIN	MIG	MIG	rys	<b>Asp</b> 85	AIG	мвр	Leu	Ala	90 90	GIY	GTÅ	Val	Ala		Ala	
	Phe	Ser	Glu	Ala	Phe	Glv	Tur	Pro	Tle		Glu	Tura	Aco	212	95	G1	
		502		100		<b>U</b>	-1-		105		GIU	Lys	veħ	110	PIO	GIU	
40	Ile	His	Lvs		Leu	Thr	Asn	Met		Glu	Asp	Ala	Glv		T.e.u	Ala	
70			115					120					125	p		7.4	
	Thr	Arg		Ala	Lys	Glu	Lys		Met	Arg	Ile	Arg		Phe	Ala	Phe	
		130			-	·	135	_		-		140					
45	Tyr	Gly	Val	Ala	Thr	Сув	Asn	Thr	Lys	Asp	Gln	Asp	Lys	Leu	Ser	Lys	
	145					150					155					160	
	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ala	Ile	Gly	Trp	Ala	Ser	Ala	
					165					170					175		
50	Leu	Val	Leu	Ser	Glu	Ile	Asn	Pro	Glu	Asn	Gln	Asp	Lys	Ile	Leu	Lys	
				180					185			•		190			
	Arg	Gly		Glu	Leu	.Gly	Gln		Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	
	_		195					200			•		205				
55	Gln		Asp	Val	Asp	Ala		Arg	Ile	Val			Gly	Ala	Val	Ala	
		210					215					220					

Thr Leu His Ser Asn Pro Glu Phe Gln Lys Gln Leu Gln Lys Ala Lys

	225					230					235					240	
5	Asp	Glu	Phe	Ala	Lув 245	Leu	Lys	Lув									
	IN	FORM	ATIO	N FO	R SE	Q ID	NO:	23:									
		(i	) SE	QUEN	CE C	HARA	CTER	ISTI	CS:								
10			(	A) L	ENGT	H:	744	base	pai	rs							
			(	B) T	YPE:	nuc	leic	aci	đ								
			(	C) S	TRAN	DEDN	ESS:	dou	ble								
			(	D) T	OPOL	OGY:	lin	ear									
15		(ii	) MO	LECU	LE T	YPE:	gen	omic	DNA								
		(iii	) HY	POTH	ETIC	AL:	МО										
		(iv	) AN	TI-S	ENSE	: NO											
		(vi	) OR														
20						ISM:			acte	r ae	roge	nes					
			-	•		N: I	FO 1	2010									
		(ix	) FE														
			_	•	•	KEY:											
25						ION:											
			QUEN														
			AAG														48
30		гλа	Lys	Arg		Leu	Ala	Leu	Cys		Ala	Ser	Leu	Phe		Val	
30	1	CCT	TTC	CCC	5	CMC	000	000	000	10	a				15		
			Phe														96
			11.0	20	Deu	V 44 4		nia	25	ABII	veb	VIG	Int	30	гув	Pro	
35	GAT	CTC	TAT		СТС	AAA	ААТ	GCA		GCC	ATC	CAT	ACT		ccc	CTC	144
			Tyr														144
	•		35			-4-		40					45			200	
	TTG	CCG	CCG	CCG	CCG	GAA	GTT	GGC	AGC	ATC	GCA	ттт		AAC	САТ	CAG	192
40			Pro														1,72
		50					55	•				60					
	GCG	ATG	TAT	GAG	AAA	GGA	CGG	CTG	TTG	CGC	AAT	ACC	GAA	CGT	GGC	AAG	240
			Tyr														2.0
45	65				_	70					75			-		80	
	CTG	GCG	GCT	GAA	GAT	GCT	AAC	CTG	AGC	GCC	GGC	GGC	GTC	GĊG	AAT	GCC	288
	Leu	Ala	Ala	Glu	Asp	Ala	Asn	Leu	Ser	Ala	Gly	Gly	Val	Ala	Asn	Ala	
					85					90					95		
50	TŢC	TCC	AGC	GCT	TTT	GGT	TCG	ccc	ATC	ACC	GAA	AAA	GAC	GCG	CCG	CAG	336
	Phe	Ser	Ser	Ala	Phe	Gly	Ser	Pro	Ile	Thr	Glu	Lys	Asp	Ala	Pro	Gln	,
				100					105					110			
			AAG														384
5 <i>5</i>	Leu	His	ГÀв	Leu	Leu	Thr	Asn	Met	Ile	Glu	Asp	Ala	Gly	Авр	Leu	Ala	
			115					120					125				

	ACC	CGC	AGC	GCG	AAA	GAG	AAA	TAT	ATG	CGC	ATT	CGC	CCG	TTT	GCG	TTC	432
		Arg															
_		130					135					140					
5	TAC	GGC	GTT	TCA	ACC	TGT	AAC	ACT	ACC	GAG	CAG	GAC	AAG	CTG	TCG	AAA	480
	Tyr	Gly	Val	Ser	Thr	Сув	Asn	Thr	Thr	Glu	Gln	Asp	Lys	Leu	ser	Lys	
	145					150					155					160	
10	AAC	GGA	TCT	TAC	CCT	TCC	GGC	CAT	ACC	TCT	ATC	GGT	TGG	GCA	ACC	GCG	528
70	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	
					165					170					175		
•		GTA															576
15	Leu	Val	Leu		Glu	Ile	Asn	Pro		Arg	Gln	Asn	Glu		Leu	Lys	
				180					185					190			
		GGC															624
	Arg	Gly	19F	GIU	Leu	GIY	GIU	200	Arg	vaı	116	Сув	_	Tyr	HIS	Trp	
20	CAG	AGC		CTC	CAT	ccc	ccc		אדא	CTC	ccc	TCC	205	CTC	CTC.	cac	670
		Ser															672
	02	210		<b>741</b>			215	9		***	O.J.	220	nia	•41	<b>V</b> 41	A.a.	
	ACC	CTG	CAT	ACC	AAC	CCG		TTC	CAA	CAG	CAG		CAG	AAA	GCA	AAG	720
25		Leu															, 20
	225					230					235					240	
	GAT	GAA	TTC	GCC	AAA	ACG	CAG	AAG	TAA								747
	Asp	Glu	Phe	Ala	Lys	Thr	Gln	Lys									
30					245												
																	•
	INI	ORM	OITE	FOF	SEÇ	D ID	NO:2	24:									
		(i)	SEÇ	-													
35			•	A) LE					acid	ls						•	
			•	3) TY													
		/ 2 2 3	•	) TO													•
40			MOI ORI				-	ein									
40		(41)		A) OF				roba	cter	- 201	-oaer	100					
			•	3) SI					CCL	aer	ogei	169					
		(xi)	SEC	•					EO I	D NO	):24:						
45	Met	Lys											Leu	Phe	Ser	Val	
40	1	-			5				•	10	–				15		
	Asn	Ala	Phe	Ala	Leu	Val	Pro	Ala	Gly	Asn	Авр	Ala	Thr	Thr	Lys	Pro	
				20					25					30	_		
50	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ala	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu	
			35					40					45				
	Leu	Pro	Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Ala	Phe	Leu	Asn	Asp	Gln	
		50					55					60					
55		Met	Tyr	Glu	Lys		Aŗg	Leu	Leu	Arg		Thr	Glu	Arg	Gly	Lys	
	65					70					75					80	
																•	

	200			•••					001		GLY	GLY	491	VIG		VIG	
					85	-1	<b>.</b>	_		90		_			95		
5	Pne	Ser	Ser		Pne	GLY	Ser	Pro		Thr	Glu	Lys	qaA	Ala	Pro	Gln	
J	_		_	100	_		_		105					110			
	Leu	His	Lys	Leu	Leu	Thr	Asn		Ile	Glu	Asp	Ala	Gly	yab	Leu	Ala	
			115					120					125				
	Thr		Ser	Ala	Lys	Glu	Lys	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	
10		130					135					140					
		Gly	Val	Ser	Thr	Сув	Asn	Thr	Thr	Glu	Gln	Asp	Lys	Leu	Ser	Lys	
	145					150					155	٠				160	
	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	
15					165					170					175		
	Leu	Val	Leu	Ala	Glu	Ile	Asn	Pro	Gln	Arg	Gln	Asn	Glu	Ile	Leu	Lys	
				180					185					190			
	Arg	Gly	Tyr	Glu	Leu	Gly	Glu	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	
20			195					200					205	_			
20	Gln	Ser	Asp	Val	Asp	Ala	Ala	Arg	Ile	Val	Gly	Ser	Ala	Val	Val	Ala	
		210					215				_	220					
	Thr	Leu	His	Thr	Asn	Pro	Ala	Phe	Gln	Gln	Gln	Leu	Gln	Lvs	Ala	Lvs	
	225					230					235					240	
25	Asp	Glu	Phe	Ala	Lys	Thr	Gln	Lys									
•					245			-,-									
3 <b>0</b>	IN	FORM	ATIO	v FOI	R SEC	r ID	NO:	25:									
			) SE						35 •								
		\-		A) LI						ra							
			•	B) T					-								
35				c) s:													
00			-	) TO					) I G								
		/ : : :	OM (	-					DATA								
		•	HY				-	Juite	DNA								
		•					10										
40		•	) ANI														
		(AT	OR.						_	_							
			-	A) OI					lla p	olant	ico)	la					
			-	3) Si		V: II	PO 14	1939									
45		(ix	) FE														
			(1	A) N2	ME/I	ŒY:	CDS										
			( I	3) LC	CATI	ON:	1	747									
	-	-	QUENC					_									
50	ATG	AAA	AAG	CGT	GTA	CTC	GCC	CTT	TGC	CTT	GCC	AGC	CTC	TTT	TCA	GTT	48
50	Met	Lys	ГЛВ	Arg	Val	Leu	Ala	Leu	Сув	Leu	Ala	Ser	Leu	Phe	Ser	Val	
	1				5					10					15		

	AGC	GCC	TTT	GCG	CTG	GTT	CCC	GCC	GGC	AAT	GAT	GCC	ACC	ACC	AAG	ccc	96
	Ser	Ala	Phe	Ala	Leu	Val	Pro	Ala	Gly	Asn	Asp	Ala	Thr	Thr	Lys	Pro	
5				20					25					30			
3		CTC															144
•	Asp	Leu		Tyr	Leu	ГЛВ	Asn	Ala	Gln	Ala	Ile	yab	Ser	Leu	Ala	Leu	
			35					40					45				
10		CCA															192
	Leu	Pro	Pro	Pro	Pro	Glu		Gly	Ser	Ile	Ala		Leu	Asn	увь	Gln	
	000	50	<b></b>	<b>636</b>			55		~~~			60					
		ATG															240
15	65 A1a	Met	TYP	GIU	гув	70	Arg	Leu	rea	Arg		Thr	Ala	Arg	GIA	-	
		GCG	GCA	CAA	CAT		AAC	CTC	<b>NCC</b>	GCG	75 ccr	ccc	CTC	666	220	80	200
		Ala															288
	200			014	85			204	561	90	Uly	GIY	, vai	ura	95	HIA	
20	ттс	TCC	GCA	GCA		GGC	TCC	CCG	ATC		GAD.	444	GAC	GCC		ccc	336
		Ser															336
				100		,			105			-,-		110	110	nia	
	CTG	CAC	AAA		CTC	ACC	AAC	ATG		GAA	GAC	GCG			CTG	GCG	384
25		His															504
			115					120			•		125	•			
	ACC	CGA	GGC	GCG	AAA	GAG	AAG	TAT	ATG	CGT	ATT	CGT	CCG	TTT	GCC	TTC	432
	Thr	Arg	Gly	Ala	Lys	Glu	Lys	Tyr	Met	Arg	Ile	Arg	Pro	Phe	Ala	Phe	
30		130					135					140					
	TAC	GGC	GTG	TCC	ACC	TGC	AAT	ACC	ACC	GAA	CAG	GAT	AAG	CTG	TCG	AAA	480
	Tyr	Gly	Val	Ser	Thr	Сув	Asn	Thr	Thr	Glu	Gln	Asp	Lys	Leu	Ser	Lys	
	145					150					155					160	
35	AAC	GGC	TCC	TAC	CCT	TCC	GGA	CAC	ACC	TCT	ATC	GGC	TGG	GCG	ACC	GCC	528
	Asn	Gly	Ser	Tyr	Pro	Ser	Gly	His	Thr	Ser	Ile	Gly	Trp	Ala	Thr	Ala	
					165					170					175	•	
		GTG															576
40	Leu	Val	Leu		Glu	Ile	Asn	Pro	Gln	Arg	Gln	Asn	Glu	Ile	Leu	Lys	
				180					185					190			
		GGC															624
	Arg	Gly		Glu	Leu	Gly	Glu		Arg	Val	Ile	Сув		Tyr	His	Trp	
45			195					200					205				
		AGC															672
	GIn	Ser	Авр	vaı	Авр			Arg	He	val	GIA		Ala	Val	Val	Ala	
		210	a.m			-	215					220					
50		CTG															720
	225	Leu	ulb	Int	иви	230	ATS	rne	GIN	GTD		Leu	GIn	ГÀв	Ala	_	
		GAG	ጥጥጥ	GCG	מממ		CAC	ממג	TDC		235					240	- 4-
		Glu															747
55	sp	JIU		*a	- 7 5	<b>411</b>	2111	-ys	•								

INFORMATION FOR SEQ ID NO:26:

		(i	) SE	QUEN	CE C	HARA	CTER	ISTI	cs:							
			(	A) L	ENGT	H: 2	48 a	mino	aci	ds						
5			(1	B) T	YPE:	ami	no a	cid								
			(1	D) T	OPOL	OGY:	lin	ear								
		(ii	) MO	LECU	LE T	YPE:	pro	tein								
10		(vi	) OR	IGIN.	AL S	OURC	E:									
10			C	A) O	RGAN	ISM:	Kle	vsie	lla	plan	tico	la				
			(1	B) S	TRAI	N: I	FO 1	4939								
		(xi	) SE	QUEN	CE D	ESCR	IPTI	ON:	SEQ	ID N	0:26	:				
15	Met	Lys	Lys	Arg	Val	Leu	Ala	Leu	Сув	Leu	Ala	Ser	Leu	Phe	Ser	Va]
	1				5					10					15	
	Ser	Ala	Phe	Ala	Leu	Val	Pro	Ala	Gly	Asn	Asp	Ala	Thr	Thr	Lys	Pro
				20					25			•		30		
20	Asp	Leu	Tyr	Tyr	Leu	Lys	Asn	Ala	Gln	Ala	Ile	Asp	Ser	Leu	Ala	Leu
			35					40					45			
	Leu	Pro	Pro	Pro	Pro	Glu	Val	Gly	Ser	Ile	Ala	Phe	Leu	Asn	Asp	Glr
		50					55					60				
25		Met	Tyr	Glu	Lys	Gly	Arg	Leu	Leu	Arg	Ala	Thr	Ala	Arg	Gly	Lye
	65					70					75					80
	Leu	Ala	Ala	Glu		Ala	Asn	Leu	Ser		Gly	Gly	Val	Ala	Asn	Ala
		_			85 		_	_		90					95	
30	Pne	Ser	Ala		Phe	Gly	Ser	Pro		Ser	Glu	Lys	Asp		Pro	Ala
	T 0	1112	*	100	<b>.</b>	mb	<b>&gt;</b>	W - 4	105	_,				1'0	_	
	Leu	птв	115	Leu	Leu	THE	Asn	120	iie	GIU	Asp	Ala		Asp	Leu	Ala
	Thr	Ara		Ala	T	C1	T 145		Wat	<b>.</b>	T1-	<b>.</b>	125	<b>51</b>	Ala	_,
35	12	130	Gry	AIG	Dyb	GIU	135	TYL	met	Arg	TIE	140	PFO	Pne	ALA	Pne
	Tvr		Val	Ser	Thr	Cvs		Thr	Thr	Glu	Gln		T.va	T.OU	Ser	T 1/0
	145	2				150				O.L.	155	op	Dy 5	Deu	261	160
40		Gly	Ser	Tvr	Pro		Glv	His	Thr	Ser		Glv	Tro	Ala	Thr	
70		-		•	165					170		1			175	
	Leu	Val	Leu	Ala	Glu	Ile	Asn	Pro	Gln		Gln	Asn	Glu	Ile	Leu	Lvs
				180					185	-				190		
45 ·	Arg	Gly	Tyr	Glu	Leu	Gly	Glu	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp
			195					200		•			205	•		-
	Gln	Ser	Asp	Val	Asp	Ala	Ala	Arg	Ile	Val	Gly	Ser	Ala	Val	Val	Ala
		210					215					220				
50	Thr	Leu	His	Thr	Asn	Pro	Ala	Phe	Gln	Gln	Gln	Leu	Gln	Lys	Ala	Lys
	225					230					235					240
	Asp	Glu	Phe	Ala	Lys	Gln	Gln-	Lys								
55					245											

INFORMATION FOR SEQ ID NO:27:

		(i)	) SE(	QUEN	CE CI	HARA	CTER:	ISTI	CS:								
_			(2	A) L	ENGT	H: '	735 1	oase	pair	rs							
5			(1	B) T	YPE:	nuc	leic	aci	£								
			((	c) s:	TRANI	DEDNI	ESS:	dou	ole								
			(1	D) T	OPOL	OGY:	line	ear									
		(11)	) MOI	LECU	LE T	YPE:	gene	omic	DNA								
10		(iii)	HYI	POTH	ETIC	AL: I	O										
		(iv)	AN'	ri-si	ENSE	: NO											
		(vi	OR:	IGIN	AL S	OURC	€:										
			(1	A) O	RGAN:	ISM:	Ser	ratia	a fic	cari	a.						
15			(1	B) S'	TRAII	N: I	AM 1	3540									
		(ix	) FE	ATUR	E:												
			(2	A) N	AME/	KEY:	CDS										
20			(1	B) L	OCAT	ION:	1	732									
20	(xi	) SE	QUEN	CE D	ESCR	IPTI	on: :	SEQ :	ID NO	0:27	:						
	ATG	AAA	AAA	ATA	TTA	TTA	GCC	ACA	TTA	AGC	TGC	GCC	GCG	TTG	ACG	CAG	48
	Met	Lys	Lys	Ile	Leu	Leu	Ala	Thr	Leu	Ser	Сув	Ala	Ala	Leu	Thr	Gln	
25	1				5					10					15		
25	TTT	TCC	TTT	GCC	GCC	AAA	GAT	GTC	ACT	ACC	CAC	CCT	GAG	GTT	TAT	TTT	96
	Phe	Ser	Phe	Ala	Ala	Lys	Asp	Val	Thr	Thr	His	Pro	Glu	Val	Tyr	Phe	
	•			20					25					30			
30	CTG	CAA	GAA	TCA	CAG	TCC	ATC	GAC	AGC	CTG	GCA	CTA	TTG	CCG	CCG	CCG	144
-	Leu	Gln	Glu	Ser	Gln	Ser	Ile	Asp	Ser	Leu	Ala	Leu	Leu	Pro	Pro	Pro	
			35					40					45				
	CCG	GCG	ATG	GAC	AGC	ATT	GAT	TTC	CTG	AAT	GAC	AAA	GCG	CAA	TAC	GAC	192
35	Pro	Ala	Met	Asp	Ser	Ile	Asp	Phe	Leu	Asn	Asp	Lys	Ala	Gln	Tyr	Asp	
		50					55					60					
	GCC	GGG	AAA	ATA	GTG	CGC	AAT	ACT	CCG	CGT	GGC	AAG	CAG	GCT	TAT	GAT	240
	Ala	Gly	Lys	Ile	Val	Arg	Asn	Thr	Pro	Arg	Gly	Lys	Gln	Ala	Tyr	yeb	
40	65					70					75					80	
	GAC	GCC	CAC	GTT	GCC	GGG	GAC	GGC	GTT	GCC	GCC	GCA	TTT	TCC	AAC	GCC	288
	Asp	Ala	His	Val	Ala	Gly	Asp	GIA	Val		Ala	Ala	Phe	Ser		Ala	
					85					90					95		
45					ATA												336
	Phe	Gly	Leu		Ile	Ala	Gln	Arg	_	Thr	Pro	Glu	Leu		Lys	Leu	
				100					105					110			
					CGT												384
50	Val	Met	_	Met	Arg	Glu	Asp		Gly	Asp	Leu	Ala		Arg	Ser	Ala	
			115					120					125				
					ATG												432
	ГÀв		His	Tyr	Met	Arg		Arg	Pro	Phe	Ala		Tyr	Asn	Glu	Ala	
<i>55</i>		130					135					140					

	ACC	TGC	CGA	CCG	GAC	GAA	GAA	AGC	ACC	CTG	TCG	AAG	AAC	GGT	TCT	TAC	480
	Thr	Сув	Arg	Pro	Asp	Glu	Glu	Ser	Thr	Leu	Ser	Lys	Asn	Gly	Ser	Tyr	
5	145					150					155					160	
J	CCT	TCC	GGC	CAT	ACC	ACC	ATC	GGC	TGG	GCG	ACC	GCG	CTG	GTG	CTG	GCT	528
	Pro	Ser	Gly	His	Thr	Thr	Ile	Gly	Trp	Ala	Thr	Ala	Leu	Val	Leu	Ala	
					165					170					175		
10	GAA	ATC	AAC	CCC	GCC	AGG	CAG	GGT	GAA	ATC	CTG	CAG	CGC	GGC	TAT	GAT	576
10	Glu	Ile	Asn	Pro	Ala	Arg	Gln	Gly	Glu	Ile	Leu	Gln	Arg	Gly	Tyr	Asp	
				180					185					190			
	ATG	GGC	CAA	AGC	CGG	GTT	ATC	TGC	GGT	TAT	CAC	TGG	CAA	AGC	GAC	GTG	624
46	Met	Gly	Gln	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	Gln	Ser	Asp	Val	
15			195					200					205				
	ACT	GCG	GCG	CGC	ATG	GCG	GCG	TCG	GCC	ATG	GTG	GCG	CGT	TTG	CAT	GCC	672
	Thr	Ala	Ala	Arg	Met	Ala	Ala	Ser	Ala	Met	Val	Ala	Arg	Leu	His	Ala	
		210					215					220					
20	GAA	CCC	ACC	TTC	GCC	GCC	CAG	CTG	CAA	AAG	GCC	AAA	GAC	GAA	TTC	AAC	720
	Glu	Pro	Thr	Phe	Ala	Ala	Gln	Leu	Gln	Lys	Ala	Lys	Asp	Glu	Phe	Asn	
	225				•	230					235					240	
	GGC	CTG	AAA	AAG	TAA												735
25	Gly	Leu	Lys	Lys													
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35		(Vi)		GINA													
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40		Lys	гав	TIE		Leu	Ala	Thr	Leu		Cys	ATA	ALA	Leu		GIn	
	1	G	<b>D</b>		5	<b>7</b>	<b>&gt;</b>	11-1	mh	10	•••		<b>-1</b> :		15	-1	
	Pne	Ser	Pne		Ald	гåа	мзр	vai		The	HIS	PFO	GIU		Tyr	Pne	
	T 0	Gln	C1	20	Cl n	50=	TIO	N a.m.	25	7	N1 -	T	T	30	Duan	Dona	
45	reu	GIII	35	Ser	GIN	ser	116	40	261	Leu	MIG	rea	45	PFO	PEO	Pro	
	Pro	Ala		Agn	Sar	Tlo	Aan		LOU	Aan	Acr	tva		Cl n	T	Nan	
		50		nop	561		55		Deu	ABII	veħ	60	VIG	GIN	ıyı	veħ	
	Δla	Gly	T.va	Tle	Val	Ara		Thr	Pro	Ara	Gly		Gla	A 1 a	Tier	Aan	
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		Ala	Hig	Val	Δla		Aan	Glv	Val	בוב		A 1 a	Dhe	502	h an		
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										20					23		
	Phe	Glv	Leu	G) u	Ile	Ala	Glp	Ara	Lve	The	Pro	Glu	Leu	Pho	Lva	Leu	
55	Phe	Gly	Leu	Glu 100	Ile	Ala	Gln	Arg	Lys. 105	Thr	Pro	Glu	Leu	Phe 110	ГÀв	Leu	

	Val Met	Lys 115	Met	Arg	Glu	Asp	Ala 120	Gly	Asp	Leu	Ala	Thr 125	Arg	Ser	Ala	
	Z		<b></b>	V-+		<b>*</b> 1-		D	Dh -		<b>5</b> 4 -		_			
5	Lys Asn 130		TYE	Mec	AEG	135	Arg	Pro	rne	Ald	140	TYP	ABN	GIU	ATA	
	Thr Cys	Arg	Pro	Авр	Glu	Glu	Ser	Thr	Leu	Ser	Lys	Asn	Gly	Ser	Tyr	
	145	_		_	150					155	-		•		160	
10	Pro Ser	Gly	His	Thr	Thr	Ile	Gly	Trp	Ala	Thr	Ala	Leu	Val	Leu	Ala	
				165					170					175		
	Glu Ile	: Asn	Pro	Ala	Arg	Gln	Gly	Glu	Ile	Leu	Gln	Arg	Gly	Tyr	Asp	
			180					185					190			
15	Met Gly	Gln	Ser	Arg	Val	Ile	Сув	Gly	Tyr	His	Trp	Gln	Ser	Asp	Val	
		195					200					205				
	Thr Ala	Ala	Arg	Met	Ala	Ala	Ser	Ala	Met	Val	Ala	Arg	Leu	His	Ala	
	210	)			•	215					220					
20	Glu Pro	Thr	Phe	Ala	Ala	Gln	Leu	Gln	Lys	Ala	Lys	Asp	Glu	Phe	Asn	
	225				230					235					240	
	Gly Leu	Lys	Lys													
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	(ii	.) MOI	-					IA	ynth	netio	DNA	4				
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3 <b>5</b>	(iv	) AN	TI-SI	ENSE:	NO :									,		
	(xi	) SE	QUEN	CE DE	ESCRI	PTIC	on: S	EQ I	D NC	29:	;					
	CCCGGCG	TCA	CCAA!	rcat <i>i</i>	AT T											21
40	INFORMA	TTON	FOR	CEO	TD N	io. 20	١.									
••		) SE														
	( -	•	A) LI												•	
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45		•	C) S1													
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	(5.5	, IOM (.	•					1 A .	usent h	atio	, DMZ					
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	GCCGGTA															21
																41

### EP 0 857 788 A2

### Claims

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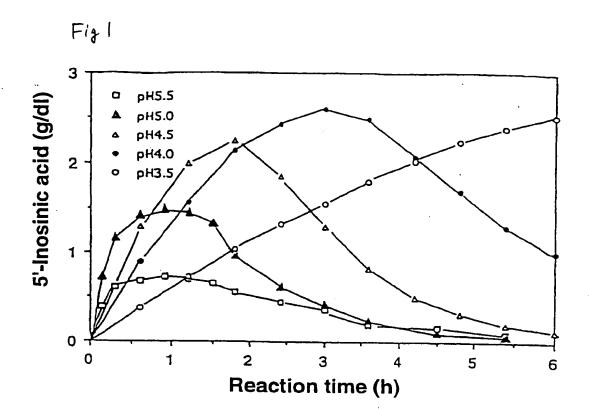
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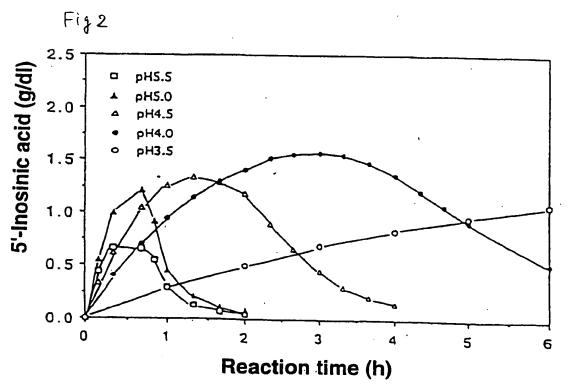
- A method for producing nucleoside-5'-phosphate ester comprising the steps of allowing an acid phosphatase having an increased affinity for the nucleoside and/or an increased temperature stability to act under a condition of pH 3.0 to 5.5 on a nucleoside and a phosphate group donor to produce nucleoside-5'-phosphate ester, and collecting it.
- 2. A method for producing nucleoside-5'-phosphate ester comprising the steps of allowing a microorganism to act under a condition of pH 3.0 to 5.5 on a nucleoside and a phosphate group donor to produce nucleoside -5'-phosphate ester, and collecting it, wherein the microorgansm is transformed with a recombinant DNA comprising a gene encoding an acid phosphate having an increased affinity for the nucleoside and/or an increased temperature stability.
- The method for producing nucleoside-5'-phosphate ester according to claims 1 or 2, wherein the Km value for the nucleoside is below 100.
  - 4. The method for producing nucleoside-5'-phosphate ester according to claims 1 or 2, wherein the acid phosphatase is stable at 50°C.
- 5. The method for producing nucleoside-5'-phosphate ester according to claims 1 or 2, wherein the acid phosphate having an increased affinity for the nucleoside is derived from abacterium belonging to the genus <u>Escherichia</u>, the genus <u>Morganella</u>, the genus <u>Providencia</u>, the genus <u>Enterobacter</u>, the genus <u>Klebsiella</u> or the genus <u>Serratia</u>.
- 6. The method for producing nucleoside-5'-phosphate ester according to claim 5, wherein the acid phosphatase comprises an amino acid sequence which is substantially identical with an amino acid sequence selected from the group consisting of sequences illustrated in SEQ ID NOs: 4, 8, 25, 27, 29 and 31 in Jequence Listing, and said acid phosphatase has a mutation which increases the affinity for the nucleoside and/or the temperature stability on an amino acid sequence selected from the group consisting of sequences illustrated in SEQ ID NOs: 4, 8, 25, 27, 29 and 31 in Sequence Listing.
  - 7. The method for producing nucleoside-5'-phosphate ester according to claim 5, wherein said mutation is selected from the group consisting of substitutions of the 63rd leucine residue, the 65th alanine residue, the 66th glutamic acid residue, the 69th aspartic acid residue, the 71st serine residue, the 72nd serine residue, the 74th glycine residue, the 85th serine residue, the 92nd alanine residue, the 94th alanine residue, the 104th glutamic acid residue, the 116th aspartic acid residue, the 130th serine residue, the 135th threonine residue, the 136th glutamic acid residue, the 151th threonine residue and/or the 153rd isoleucine residue with another amino acid in SEQ ID NO: 8 in Sequence Listing.
  - 8. The method for producing nucleoside-5'-phosphate ester according to claims 1 or 2, wherein said phosphate group donor is selected from the group consisting of polyphosphoric acid or a salt thereof, acetylphosphoric acid or a salt thereof, acetylphosphoric acid or a salt thereof.
    - 9. A mutant acid phosphatase comprising an amino acid sequence which is substantially identical with an amino acid sequence selected from the group consisting of sequences illustrated in SEQ ID NOs: 4, 8, 25, 27, 29 and 31 in Sequence Listing, and said acid phosphatase has a mutation which increases the affinity for the nucleoside and/or the temperature stability on an amino acid sequence selected from the group consisting of sequences illustrated in SEQ ID NOs: 4, 8, 25, 27, 29 and 31 in Sequence Listing.
    - 10. A mutant acid phosphatase according to claim 9, wherein said mutation is selected from the group consisting of substitutions of the 63rd leucine residue, the 65th alanine residue, the 66th glutamic acid residue, the 69th aspartic acid residue, the 71st serine residue, the 72nd serine residue, the 74th glycine residue, the 85th serine residue, the 92nd alanine residue, the 94th alanine residue, the 104th threonine residue, the 116th aspartic acid residue, the 130th serine residue, the 135th threonine residue, the 136th glutamic acid residue, the 151st threonine residue and/or the 153rd isoleucine residue with another amino acid in SEQ ID NO: 8 in Sequence Listing.
    - 11. A gene coding for the acid phosphatase as defined in claim.9
    - 12. A recombinant DNA comprising the gene as defined in claim 11.

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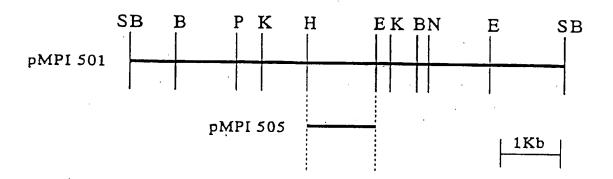
13. A microorganism harboring the recombinant DNA as defined in claim 12.

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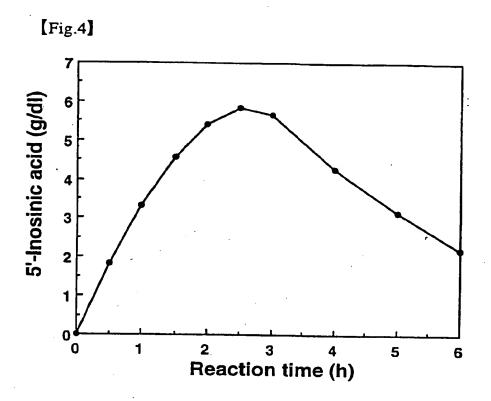


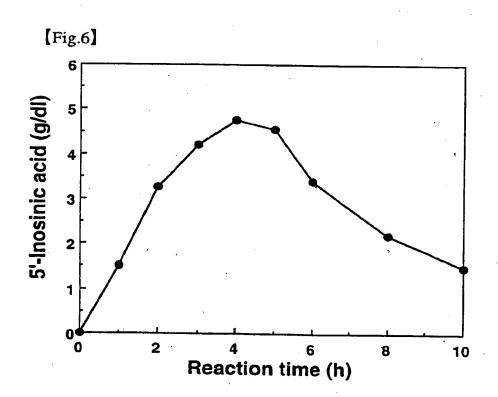


# F 1 G. 3

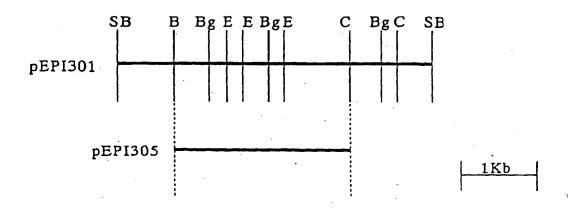


SB: Sau 3AI / Bam HI junction B: Bam HI E: Eco RI K: Kpn I H: Hind III N: Nco I P: Pst I

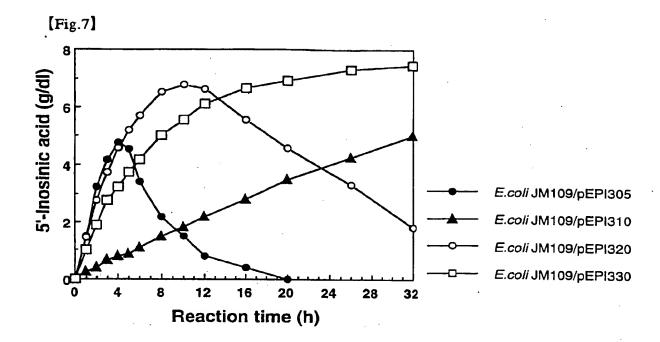


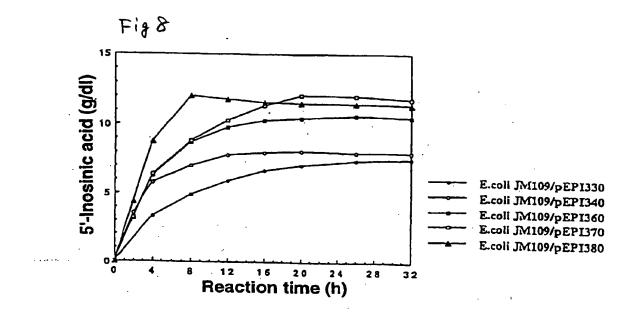


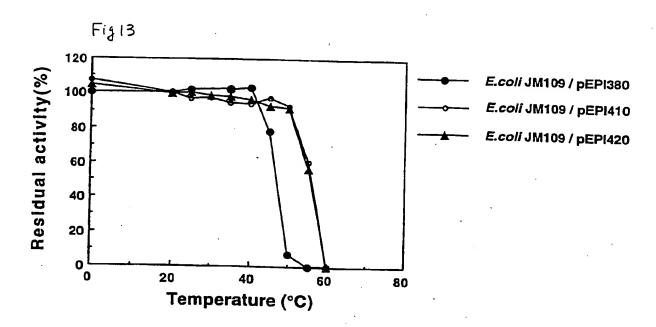
# FIG. 55



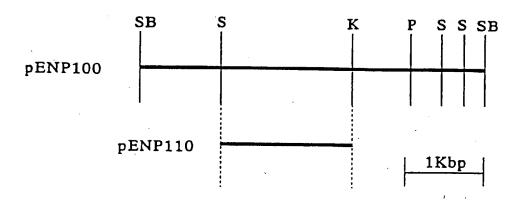
SB: Sau3AI / BamHI junction B: BamHI Bg: BglII C: ClaI E: EcoRI





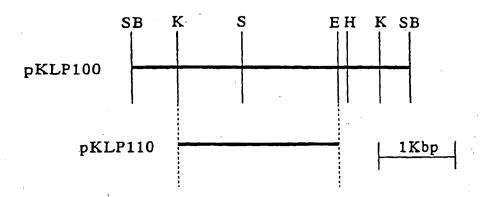


### F 1 G. 9



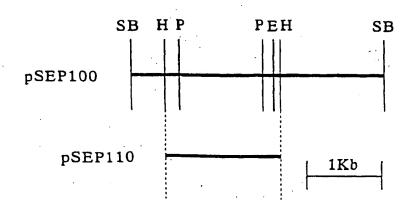
SB: Sau3AI / BamHI junction K: KpnI P: PstI S: SalI

## F I G. 10



SB: Sau3AI / BamHI junction E: EcoRI H: HindIII K: KpnI S: SacI

## F I G. 11



SB: Sau3Al / BamHl junction E: EcoRl H: HindIII P: PstI

## F I G. 12

E. aerogenes E. blattae K. planticola	1: MKKRVLAVCFAALFSSQALALVATGNDTTTKPDLYYLKNSEAINSLALLP	50 50
M. morganii	1:MKKNIIAGCLFSLFSLSALAAIPAGNDATTKPDLYYLKNEQAIDSLKLLP	50
P. stuartii		50
S. ficaria	·	46
0.1.001.1	*** * * * * * * * * * * * * * * * * * *	- •
	•	
E. aerogenes	51:PPPEVGSIAFLNDQAMYEKGRLLRNTERGKLAAEDANLSAGGVANAFSSA 1	00
E. blattae		00
K. planticola		00
M. morganii		00
P. stuartii		00
S. ficaria	· · · · · · · · · · · · · · · · · · ·	96
J. IICalla	*** ** * * * * * * * * * * * * * * * * *	, J
E. aerogenes	101:FGSPITEKDAPQLHKLLTNMIEDAGDLATRSAKEKYMRIKPFAFYGVSTC 1	50
E. blattae	" - · · · · · · · · · · · · · · · · · ·	50
K.planticola		50
M. morganii		50 50
P. stuartii		50
S. ficaria		46
S. HCarta	97.FGLEINURAIFELFALTMAMALUNGULAIRANAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	40
E. aerogenes	151:NTTEQDKLSKNGSYPSGHTSIGWATALVLAEINPQRQNEILKRGYELGES 2	00
E. blattae		00
K. planticola		00
M. morganii	• •	00
P. stuartii		00
S. ficaria		96
3. IICaria	1 Cypmulynyulauynnntathatinathatinathaticadula.ipi	90
E. blattae	201:RVICGYHWQSDVDAARVVGSAVYATLHTNPAFQQQLQKAKAEFAQHQKK 2	49
K. planticola		49 48
M. morganii		40 49
E. aerogenes		48
P. stuartii	· · · · · · · · · · · · · · · · · · ·	48
S. ficaria		44
S. IICATIA	197:KV1CGINNQSUVIAAKMAASAMVAKLNAEPIPAAQLQKAKUEP-NGLKK 2	44
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